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(54) Title: PROTOZOAN EXPRESSION SYSTEMS FOR LYSOSOMAL STORAGE DISEASE GENES

(57) Abstract: Methods and devices are provided for expressing and secreting gene products from protozoa. The methods and devices are useful for expressing and isolating lysosomal storage disease enzymes from protozoa grown in culture, and particularly from trypanosomatids. The post-translational modification of isolated expression products can be adapted for administration to mammalian organisms. In addition, expression products can be isolated from serum free cultures thereby avoiding contamination by infectious agents such as prions. The methods and devices are also useful for delivering expression products such as lysosomal storage disease enzymes to mammalian organisms *in vivo*.

PROTOZOAN EXPRESSION SYSTEMS FOR LYSOSOMAL STORAGE DISEASE GENES

RELATED APPLICATIONS

[0001] This application claims priority to, and the benefit of U.S.S.N. 60/250,446 filed on November 30, 2000, U.S.S.N. 60/250,444 filed on November 30, 2000, and U.S.S.N. 60/290,281 filed on May 11, 2001, the disclosures of which are incorporated by reference herein in their entirety.

FIELD OF THE INVENTION

[0002] The invention relates generally to the field of pharmaceutical devices. The invention relates to methods and compositions for producing and delivering pharmaceutical products to a patient. In particular, the invention relates to methods and compositions for producing pharmaceutical products to treat lysosomal storage diseases, and to methods for administering pharmaceutical products to patients suffering from lysosomal storage diseases.

BACKGROUND

[0003] Lysosomal Storage Diseases (LSD) are caused by one or more metabolic errors that result in an abnormal accumulation or processing of material in lysosomes. Lysosomal storage diseases are usually inherited, and can be severely debilitating. At least 50 different types of lysosomal storage diseases have been identified in humans and animals. Each disease is characterized by a unique set of symptoms resulting from a loss of normal lysosomal function affecting one or more areas of the body of an individual. Symptoms may include mental and/or physical disabilities and often result in a shortened lifespan.

[0004] Most lysosomal storage diseases are caused by a deficiency in an enzyme that processes one or more products of cellular metabolism. Each disease is

characterized by a defect in a specific enzyme. Usually, the enzyme defect results from a mutation that either deletes the gene encoding the enzyme, reduces expression of the enzyme, or affects the normal function of the enzyme.

[0005] There is therefore a need in the art for devices and methods to correct the enzyme deficiencies associated with lysosomal storage diseases.

SUMMARY

[0006] The invention provides methods and devices for producing a gene product for administration to a mammal. According to the invention, a gene product is produced in a unicellular organism that is adapted for survival or growth in a host cell, tissue or organism. The gene product is preferably processed so that it is compatible with the host.

[0007] Methods and devices of the invention are useful to provide an enzyme or enzyme product to a host. Methods of the invention are particularly useful to provide an enzyme or enzyme product to the lysosomes of a patient suffering from a lysosomal storage disease.

[0008] In one aspect of the invention, a gene product is synthesized using a microorganism in an *in vitro* culture. In another aspect of the invention, a gene product is synthesized *in vivo* by a microorganism that has been administered to a host cell, tissue or organism.

[0009] A preferred microorganism of the invention is a protozoan. In a preferred embodiment, a protozoan is modified to reduce its virulence.

[0010] Preferred gene products of the invention are proteins. A preferred protein has a biological function similar to the function that is missing in a patient with a lysosomal storage disease. A most preferred protein is an enzyme that is defective in a patient suffering from a lysosomal storage disease.

[0011] According to one aspect of the invention, a gene product is expressed using a microorganism that is adapted to survive and/or grow in a host. In one embodiment, a gene product is encoded on extrachromosomal nucleic acid in the

microorganism. In another embodiment, a gene product is encoded on chromosomal nucleic acid in the microorganism.

BRIEF DESCRIPTION OF THE FIGURES

[0012] FIG. 1A shows plasmid pIR1SAT, an expression plasmid.

[0013] FIG. 1B shows plasmid pSPITS, a Signal Peptide Increased Translation and Secretion plasmid.

[0014] FIG. 2 shows expression of GLA in promastigote cultures of *L. mexicana* cell lines, the amount of GLA released into the culture medium and the amount of cellular GLA are shown for wild-type *L. mexicana* (*L. mex*), three independent *L. mexicana* cells lines transfected with a GLA expression construct (cell lines 150.1, 156.1, and 156.2), and a negative control transfected with a βGUS expression construct (cell line 168.1).

[0015] FIG. 3 shows the GLA expression results of FIG. 2, and also shows, for each cell line, the amount of G6PDH (a cytosolic marker) present in the medium relative to the amount of G6PDH associated with the cells.

[0016] FIG. 4 shows expression of βGUS in promastigote cultures of *L. mexicana* cell lines, the amount of βGUS released into the culture medium and the amount of cellular βGUS are shown for wild-type *L. mexicana* (*L. mex*), three independent *L. mexicana* cells lines transfected with a βGUS expression construct (cell lines 168.1, 168.2, and 169.1), and a negative control transfected with a GLA expression construct (cell line 150.1). The expression of βGUS from an episomal construct in *L. major* is also shown (cell line NeoGus).

[0017] FIG. 5 shows the βGUS expression results of FIG. 4, and also shows, for each cell line, the amount of G6PDH (a cytosolic marker) present in the medium relative to the amount of G6PDH associated with the cells.

[0018] FIG. 6 shows expression of GLA in amastigote cultures of *L. mexicana* cells lines, the amount of GLA released into the culture medium and the

amount of cellular GLA are shown for wild-type *L. mexicana* (*L. mex*), three independent *L. mexicana* cells lines transfected with a GLA expression construct (cell lines 150.1, 156.1, and 156.2), and a negative control transfected with a βGUS expression construct (cell line 168.1).

[0019] FIG. 7 shows expression of βGUS in amastigote cultures of *L. mexicana* cell lines, the amount of βGUS released into the culture medium and the amount of cellular βGUS are shown for wild-type *L. mexicana* (*L. mex*), three independent *L. mexicana* cells lines transfected with a βGUS expression construct (cell lines 168.1, 168.2, and 169.1), and a negative control transfected with a GLA expression construct (cell line 150.1). The expression of bacterial βGUS from an episomal construct in *L. major* is also shown (cell line NeoGus).

[0020] FIG. 8 shows GLA expression in independent isolates of *L. mexicana* cell lines transfected with a GLA expression construct.

[0021] FIG. 9 shows βGUS expression in independent isolates of *L. mexicana* cell lines transfected with a βGUS expression construct.

[0022] FIG. 10A shows a time course of GLA release into the medium for an *L. mexicana* cell line transfected with a GLA expression construct.

[0023] FIG. 10B shows a time course of βGUS release into the medium for an *L. mexicana* cell line transfected with a βGUS expression construct.

[0024] FIG. 11 shows GBA expression in promastigote *Leishmania* lines containing GBA-SAP constructs; LM16 is wild type *L. mexicana;* 367.1 and 377.1 are lines containing GBA cassettes cloned into the Xba or Bgl site of pIR1-SAT.

[0025] FIG. 12 shows GBA expression in amastigote *Leishmania* lines containing GBA-SAP constructs; LM16 is wild type *L. mexicana*; 367.1 and 377.1 are lines containing GBA cassettes cloned into the Xba or Bgl site of pIR1-SAT.

[0026] FIG. 13 shows HexA expression in independently isolated *Leishmania* strains; HexA activity is measured using substrate MUGS; promastigote lines containing the HexA, HexB, or HexA and HexB genes are indicated.

[0027] FIG. 14 shows HexAB expression in independently isolated *Leishmania* strains; HexAB activity is measured using substrate MUG.

DETAILED DESCRIPTION

[0028] The present invention provides methods and compositions for producing a gene product for a use in a pharmaceutical or therapeutic treatment. The invention is useful to produce a gene product in a form that is biologically compatible with an organism to which the gene product is intended to be administered. The invention is also useful to deliver a gene product directly to a patient. The invention is particularly useful to treat a patient with a lysosomal storage disease.

[0029] In general, methods of the invention involve expressing a gene product in a unicellular organism, preferably a protozoan. In one aspect of the invention, the gene product is obtained or purified from an *in vitro* culture of the unicellular organism. In preferred embodiments, the gene product is a therapeutic gene product that is administered to a patient. In another aspect of the invention, the unicellular organism is itself administered to a patient, and acts as a delivery device for the gene product.

[0030] Protozoans are eukaryotic organisms and the post-translational modifications of gene products expressed in protozoans are characteristic of eukaryotic post-translational modifications (e.g. patterns of protein glycosylation). Accordingly, protozoans are useful vehicles for expressing gene products to be administered to eukaryotes, including mammals.

[0031] Useful protozoans include the groups of protozoans listed in Table 1. Useful protozoans include free-living protozoans and protozoans that interact with a host organism during at least part of their life cycle. This interaction may be parasitic, symbiotic, or commensal. Parasitic organisms are preferably non-pathogenic or avirulent. Particularly useful protozoans are those that are adapted for growth and survival in mammals. These organisms are characterized by post-translational modification that is biologically compatible with their mammalian host. Accordingly, a therapeutic gene product that is expressed in one of these organisms will be suitable for administration to a mammalian host.

[0032] The predominant N-linked carbohydrate found on glycoproteins produced by Trypanosomatids are of the high mannose type. The number of mannose residues and their configuration vary amongst members of this family. This variation in mannose structure may be a consideration in choosing the appropriate strain for *in vitro* and *in vivo* production of LSD proteins. In one embodiment, preferred trypanosomatid species for protein production are those that yield proteins with carbohydrates conferring the greatest affinity for the mannose receptor thereby directing LSD proteins to the lysosomes of cells of the macrophage lineage. Candidate species can be evaluated by assaying uptake of test proteins in J774-E cells, a macrophage cell line that expresses the mannose receptor as described in Example 11.

[0033] Another consideration is the ability of the expressed enzyme to serve as a substrate for mannose-6-phosphate modification. In one embodiment of the invention, a high mannose carbohydrate can serve as a substrate for the GlcNac phosphotransferase that is the first enzyme in the two step pathway for M6P modification. According to the invention, when it is desirable to modify an expression product for generating uptake competency via interaction with an M6P receptor, it is useful to consider the distinct structures of high mannose carbohydrates associated with expression in different protozoan organisms.

[0034] According to the invention, protein modification can be done *in vitro* after purification of the protein, or *in vivo* by engineering a trypanosomatid organism to express a relevant genes of the modification pathway.

[0035] According to the invention, LSD proteins secreted from *Leishmania* contain carbohydrate with terminal mannose residues, as shown in Example 9. In one aspect of the invention, other enzymes involved in glycosylation are expressed in a suitable trypanosomatid such as *Leishmania* in order to tailor carbohydrate patterns for desirable properties. For example, expression of a *T. cruzi* trans-sialidase in *Leishmania* can be useful to produce recombinant LSD proteins with carbohydrates containing terminal sialic acid residues. This modification could also have the beneficial property of increasing the half-life of the LSD protein in the circulation of patients.

Table 1 Useful protozoans.

Group	Common Name	Typical representatives	Habitats	Common diseases
Mastigophora	Flagellates	*Trypanosoma, Giardia, Leishmania*	Freshwater; parasites of animals	African sleeping sickness, giardiasis, leishmaniasis
Sarcodina	Amoebas	*Amoeba, Entamoeba*	Freshwater and marine; animal parasites	Amoebic dysentery (Amebiasis)
Ciliophora	Ciliates	*Balantidium, Paramecium*	Freshwater and marine; animal parasites; rumen	Dysentery
Sporozoa	Sporozoans	*Plasmodium, Toxoplasma*	Primarily animal parasites; insects (vectors for parasitic diseases)	Malaria, toxoplasmosis

[0036] The invention provides methods and compositions for engineering protozoa to express gene products that are of therapeutic interest. Accordingly, methods of the invention are useful to overcome general problems associated with the development of a production system for a gene product that is to be used as a pharmaceutical or therapeutic agent. In particular, the invention provides i) a gene product in a form that is biologically compatible with the intended patient tissue or organism, ii) a purified gene product that is free of pathogens including prions, and iii) a delivery system that can target patient cells, fluids, and/or tissues. In preferred embodiments, methods and compositions of the invention are used in treatments for lysosomal storage diseases. According to the invention, a lysosomal storage disease (LSD) gene is expressed in a protozoan organism. In one embodiment, the LSD gene product is purified from the organism grown in culture, and the gene gene product is administered to a patient suffering from the corresponding LSD. According to the invention, a LSD gene product may be purified from cells, from a cell culture supernatant, or both. In another embodiment, the protozoan expressing the LSD gene is administered to the patient suffering from the LSD, and LSD gene expression in the protozoan rescues the lysosomal storage defect. Other aspects of the invention will be apparent from the present disclosure.

I. Unicellular Organisms

[0037] According to the invention, a gene product is expressed in a unicellular organism. Preferred unicellular organisms of the invention are eukaryotic organisms, and most preferably protozoans. In one embodiment, a preferred organism is non-pathogenic or avirulent. Accordingly, commensal and symbiotic organisms are useful organisms. In another embodiment, a preferred organism is an attenuated organism. An attenuated organism is preferably an organism with reduced viability in its host. Accordingly, an attenuated pathogen or parasitic organism may be useful in a method of the invention. In an alternative embodiment of the invention, a pathogenic protozoan can be used to deliver a therapeutic protein, preferably an LSD enzyme, to a patient, if the benefit of providing the therapeutic protein outweighs the side effects of the pathogen.

[0038] As used herein, the term protozoan includes any unicellular protozoan suitable for use in the devices and methods of the present invention. Haploid and diploid, including asexual diploid, protozoa are contemplated. Particularly preferred protozoa are parasitic protozoa, especially if they are non-pathogenic or avirulent, either naturally or through genetic manipulation. Set forth below is a non-limiting list of those unicellular protozoa contemplated to be within the scope of the present invention. Also set forth below is a discussion of generally preferred features and characteristics of the unicellular organisms most suitable for use in the present invention.

[0039] As discussed herein, currently preferred protozoans are Trypanosomatidae, including *Leishmania* and *Viannia* species. Currently preferred genera include *Leishmania*, *Trypanosoma*, *Toxoplasma*, *Plasmodium*, *Eimeria*, *Cryptosporidia*, *Giardia*, *Entamoeba*, Acanthamoeba, *Naegleria*, *Microspoidia*, and *Trichomona*. Most currently preferred are the species *L. major*, *L. tropica*, *L. aethiopica*, *L. enrietti*, *L. panamaenisis*, *L. guyanensis*, *L. donovani*, *L. chagasi*, *L. infantum*, *L. tarentolae*, *L. mexicana*, *L. amazonesis*, *L. braziliensis*, *T. cruzi*, *T. brucei* and members of the trypanosomatid genus *Endotrypanum* such as *E. monterogei* and *E. schaudinni*. Other currently preferred species are *T. gondii*, *G. lamblia*, *T. vaginalis* and *T. foetus*.

[0040] A particularly preferred diploid genus is *Leishmania*. A particular advantage of expressing LSD genes in *Leishmania* is that the glycosylation of proteins in *Leishmania* is believed to occur without significant addition of terminal sialic acid residues. The resulting availability of mannose residues will permit uptake of the LSD proteins by macrophages without further post-translational modification, because the presence of mannose residues targets a protein to mammalian lysosomes and to macrophages. According to the invention, a preferred expression system for *Leishmania* is based on ribosomal gene promoters under the control of RNA polymerase 1 (PolI). A PolI expression construct of the invention may be episomal (e.g. expression of HexB was obtained from an episomal PolI expression construct) or chromosomal. In one embodiment, chromosomal expression is obtained by integrating a PolI expression construct into a ribosomal gene via homologous recombination using a linearized PolI expression vector. In an alternative embodiment, a PolI expression construct is integrated (either randomLy or via homologous recombination) at a non-ribosomal genetic locus, and the recombinant gene product (e.g. an LSD enzyme) can be expressed even after the recombinant cells reach stationary phase.

[0041] A particularly preferred haploid genus is *Toxoplasma*. *Toxoplasma* is an obligate intracellular parasite. It is well known that *Toxoplasma* is culturable. All of the known protein-coding genes are present in single-copy. Gene expression in *Toxoplasma* is apparently conventional; that is, promoters are defined and thematically similar to higher eukaryotes. (See, for example, 1995 Molecular Approaches to Parasitology, pp. 211-225, Boothroyd et al. (eds. J.C. Boothroyd & R. Komuniecki; J. Wiley & Sons, N.Y.) An especially preferred species that is well-characterized is *T. gondii*. Toxoplasma is distributed world-wide and resides in various cells, tissues and fluids of the host. During certain stages, the organism can be found in the central nervous system, skeletal and cardiac muscles, and visceral organs and tissues. Domestic cats are important reservoirs for human infection of this organism. For example, human infection can be transmitted in a variety of ways: handling infected cat feces; ingestion of meat from infected animals such as pork and lamb; transplacental transmission; transfusion with infected blood; and, via organ transplantation from infected donors. While toxoplasmic encephalitis is the most common opportunistic

parasitic infection of the central nervous system in patients with AIDS, most individuals can harbor infection asymptomatically. *Toxoplasma* infection has also been shown to affect rat behavior indicating that it acts at the level of the CNS. According to the invention, *Toxoplasma* is useful for *in vivo* delivery of one or more expression products across the blood brain barrier.

[0042] Other suitable protozoans known to have human hosts include: *Entamoeba histolytica, Entamoeba dispar, Entamoeba hartmanni, Entamoeba coli, Entamoeba polecki, Endolimax nana, Iodamoeba buetschlii, Naegleria fowleri, Acanthamoeba species, Dientamoeba fragilis, Giardia lamblia, Chilomastix mesnili, Trichomonas vaginalis, Pentatrichomonas hominis, Enteromonas hominis, Balantidium coli, Blastocystis hominis, Isospora belli, Sarcocystis species, Cryptosporidium parvum, Enterocytozoon bieneusi, Toxoplasma gondii, Plasmodium vivax, Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium species, Babesia microti, B. equi, B. bigemina, Trypanosoma b. gambiense, T.b. rhodesiense, T. cruzi, T. rangeli,* and *Pneumocystis carinii.*

[0043] In one aspect of the invention, a protozoan expressing a gene product is grown *in vitro*, and the gene product is obtained from the *in vitro* culture. In a preferred embodiment, an expressed gene product is released or secreted into the culture medium. The gene product is then obtained or purified from the culture medium. In an alternative embodiment, an expressed gene product is retained within the microorganism, and the gene product is obtained or purified from the microorganism.

[0044] In another aspect of the invention, a protozoan expressing a gene product is administered to a host organism, and the gene product is expressed by the protozoan *in situ* in the host. In a preferred embodiment, the gene product is secreted from the protozoan. In an alternative embodiment, the gene product is bound to the external membrane of the protozoan. In yet a further embodiment, the gene product remains inside the protozoan, and metabolizes one or more substrates that diffuse into or are transported into the protozoan from the host. Reaction product then diffuses out, or is transported out from the protozoan to the host.

A. Organisms useful for *in vitro* production

[0045] Preferred organisms for *in vitro* growth and gene expression are microorganisms that grow in culture. A preferred microorganism is a protozoan that grows to high density *in vitro*. This is useful to obtain large amounts of the expressed gene product from the *in vitro* culture.

[0046] Preferred protozoa for *in vitro* production and secretion generally include trypanosomatids, and preferably include *Leishmania* species such as *L. tarentolae,* and *L. mexicana*, and other protozoa such as *Crithidia* which are non-pathogenic and can be grown *in vitro*. Different protozoa can be used for the *in vitro* production of expression products such as LSD enzymes in order to obtain proteins with different patterns of post-translational modification. According to the invention, different post-translational modifications may be adapted for different uses, including administering an isolated or purified expression product to mammalian organisms, as discussed below. Examples of groups of protozoa with different patterns of post-translational modification include *Trypanosoma*, *Leptomonas*, and *Herpetomonas*; *Crithidia* and *Leishmania enrietti*; *Leishmania braziliensis*, *L. amazonensis*, and *Blastocrithidia*; *Leishmania major* and *L. chagasi*; and *Leishmania tarentolae*. The pattern of post-translational modification in *Trypanosoma*, *Leptomonas*, and *Herpetomona* is similar to the pattern of post-translational modification in mammals, and recombinant proteins (e.g. LSD enzymes) isolated from *Trypanosoma*, *Leptomonas*, and *Herpetomona* are preferred substrates for phosphorylation. However, proteins isolated from other protozoa may also be phosphorylated. According to the invention, recombinant proteins can be isolated or purified from recombinant trypanosomatids (from cells or from a cell culture supernatant if the recombinant protein product is secreted) via affinity purification of a protein with a high mannose carbohydrate using for example lectin affinity chromatography (e.g. based on the interaction of mannose with ConA).

[0047] In a preferred embodiment of the invention, a microorganism is grown in culture in the absence of growth supplements derived from a mammalian source. Accordingly, the *in vitro* culture, and any gene product purified therefrom, will

be free of any mammalian contaminant. In particular the culture and purified gene product will be prion free.

[0048] A preferred prion free culture medium is a synthetic medium that does not contain serum, lymphokines or any other growth factors derived from serum. A most preferred medium is serum free M199. A most preferred organism that grows in serum free media is *Leishmania tarentolae*. *Leishmania tarentolae* was isolated from a reptile, and grows in brain heart infusion in the absence of serum or other growth supplement derived from mammals, such as fetal bovine serum or bovine embryonic fluid. However, *Leishmania tarentolae* is preferably grown in serum free synthetic medium, to avoid contamination of the culture with disease agents such as prions, which may be present in brain heart infusion and in serum. It is expected that other protozoan organisms that are free-living, or include a non-mammalian host in their life-cycle, will be readily grown in serum free synthetic culture medium. An advantage of using a protozoan expression system is that protozoan organisms do not produce prions.

[0049] Leishmania *tarentolae* is a most preferred species for the expression system, because, in addition to growing in serum free medium, it also grows to high density in an *in vitro* culture. This is particularly advantageous both from a cost standpoint as well as from a safety standpoint, because proteins produced in this culture system will be produced in the absence of any mammalian materials (thereby preventing contamination of the protein preparation by prions or other infective agents such as retroviruses). LSD gene expression constructs could be readily beintroduced into *L. tarentolae* to obtain active enzyme.

[0050] In an alternative embodiment, a prion free culture medium may include a lymphokine or other growth factor, provided that the lymphokine or growth factor is produced recombinantly and is not obtained from a mammalian serum. Accordingly, a protozoan that requires one or more lymphokines or other growth factors may be grown in a prion free culture medium. A gene product obtained from this culture will be prion free, and also free of any other potentially harmful mammalian contaminant (such as retroviruses etc.)

[0051] In other embodiments of the invention, a culture of recombinant protozoa grown in medium containing serum is diluted into serum free medium and grown for several generations. An expression product, preferably a LSD gene product, can be purified from the serum free culture. Such serum free growth and purification methods are illustrated in Example 5 and can be applied to protozoa other than *Leishmania tarentolae*. These serum free growth methods are particularly useful for preparing prion free expression products from other *Leishmania* species and other preferred organisms of the invention.

[0052] In one embodiment of the invention, a protozoan for *in vitro* gene product expression is selected such that it will not infect the mammal to which the purified gene product will be administered. In a preferred embodiment, the protozoan is an attenuated organism that is not pathogenic for humans. In a most preferred embodiment, the protozoan is not a human pathogen or parasite.

B. Organisms useful for *in vivo* production

i. General features

[0053] Preferred organisms for *in vivo* gene product expression are protozoa that survive or grow in the host to which the gene product is to be delivered. In a preferred embodiment, the protozoan is avirulent, and more preferably non-pathogenic. Useful protozoa are symbiotic or commensal organisms. Alternatively, an attenuated parasitic organism is useful to express a gene product in a host.

[0054] In one aspect of the invention, a protozoan persists in the host in order to achieve long term delivery of the expression product to the host, preferably between 1 year and several decades.

[0055] In another aspect of the invention, a protozoan only survives in the host for a short period of time, preferably between approximately 1 hour and approximately 7 days, in order to have short term delivery of the expression product and in order to minimize any long term effects caused by the presence of the protozoan in the host. Alternatively, a protozoan of the invention survives for an intermediate period of

time in the host, preferably between approximately 1 month and approximately 12 months.

[0056] Preferred attenuated organisms are conditionally defective organisms, such as auxotrophic organisms. A useful auxotrophic organism is a DHFR-TS⁻ *Leishmania* species. For example, a DHFR-TS⁻ *L. major* strain has a reduced half-life in mice, and reduced growth in macrophages.

[0057] Other examples of attenuated organisms are also useful. In general, an attenuated protozoan is generated by functionally disabling a gene required for growth and replication in the host. Examples of such genes are known in the art, for example the lpgl⁻ gene of Leishmania *major*. Knockouts of other genes such as the gp63 surface protease gene also produce attenuated organisms.

[0058] In an alternative embodiment of the invention, a protozoan is engineered to contain a suicide gene such as the HSV-thymidine kinase gene. In the presence of the appropriate drug (for example gancyclovir), the protozoan is killed. Accordingly, the persistence of a protozoan with a suicide gene can be controlled directly.

ii. Organisms for treating lysosomal storage diseases

[0059] Lysosomal storage diseases typically cause tissue specific symptoms, even though the defect is usually present in all cells of the body. For example, Gaucher disease is manifested primarily in the liver, where defective lysosomal processing in macrophages is deleterious. Many LSDs cause neurological defects due to a defective processing of lipids that are characteristic of the CNS (e.g. sphingolipids and gangliosides).

[0060] Accordingly, in one aspect of the invention, a useful protozoan is a tissue specific protozoan that will specifically target the tissue where the LSD gene defect is manifested. For example, *Toxoplasma* species are useful to target neuronal tissue, and *Leishmania* species are useful to target macrophages in the liver. In one embodiment, *Leishmania donovani* is expected to be a particularly useful protozoan for delivering an LSD gene product to macrophages in the liver.

[0061] Other useful protozoa are characterized by the following tissue specificities, and are useful to deliver LSD gene products to these tissues: skin: *Leishmania*; eye: *Acanthamoeba*; mouth: Amoebae and flagellates (usually non-pathogenic); gut: *Giardia, Entamoeba* (and invasion to liver), *Cryptosporidium, Isospora, Balantidium*; G.I. tract: *Trichomonas*; bloodstream: *Plasmodium, Trypanosoma*; spleen: *Leishmania*; liver: *Leishmania, Entamoeba*; muscle: *Trypanosoma cruzi*; CNS: *Trypanosoma, Naegleria, Toxoplasma, Plasmodium.*

[0062] In an alternative embodiment of the invention, a LSD gene is not targeted to a particular tissue, but is expressed in a protozoan that is present throughout the body. For example, many *Leishmania* species are useful to deliver a gene product to macrophages throughout the body, and more particularly to the lysosomes of the macrophages.

II. Gene Products

[0063] While methods and compositions of the invention are useful for producing and delivering any therapeutic gene product, the invention is particularly useful for gene products that overcome enzymatic defects associated with lysosomal storage diseases.

[0064] Preferred LSD genes are shown in Table 2. In a preferred embodiment, a wild-type LSD gene product is delivered to a patient suffering from a defect in the same LSD gene. In particular, a human LSD gene or enzyme is preferably delivered to a human patient. In alternative embodiments, a functional sequence or species variant of the LSD gene or protein is used. Preferred species variants are mammalian and include mouse LSD genes and proteins. In further embodiments, a gene coding for a different enzyme that can rescue an LSD gene defect is used according to methods of the invention.

Table 2. Lysosomal Storage Diseases and associated enzyme defects

A. Glycogenosis Disorders		
Disease Name	Enzyme Defect	Substance Stored
Pompe Disease	Acid-a1, 4-Glucosidase	Glycogen α 1-4 linked Oligosaccharides
B. Glycolipidosis Disorders		
Disease Name	Enzyme Defect	Substance Stored
GM1 Gangliodsidosis	β-Galactosidase	GM_1 Ganliosides
Tay-Sachs Disease	β-Hexosaminidase A	GM_2 Ganglioside
GM2 Gangliosidosis: AB Variant	GM_2 Activator Protein	GM_2 Ganglioside
Sandhoff Disease	β-Hexosaminidase A&B	GM_2 Ganglioside
Fabry Disease	α-Galactosidase A	Globosides
Gaucher Disease	Glucocerebrosidase	Glucosylceramide
Metachromatic Leukodystrophy	Arylsulfatase A	Sulphatides
Krabbe Disease	Galactosylceramidase	Galactocerebroside
Niemann-Pick, Types A and B	Acid Sphingomyelinase	Sphingomyelin
Niemann-Pick, Type C	Cholesterol Esterification Defect	Sphingomyelin
Nieman-Pick, Type D	Unknown	Sphingomyelin
Farber Disease	Acid Ceramidase	Ceramide
Wolman Disease	Acid Lipase	Cholesteryl Esters
C. Mucopolysaccharide Disorders		
Disease Name	Enzyme Defect	Substance Stored
Hurler Syndrome (MPS IH)	α-L-Iduronidase	Heparan & Dermatan Sulfates
Scheie Syndrome (MPS IS)	α-L-Iduronidase	Heparan & Dermatan, Sulfates
Hurler-Scheie (MPS IH/S)	α-L-Iduronidase	Heparan & Dermatan Sulfates
Hunter Syndrome (MPS II)	Iduronate Sulfatase	Heparan & Dermatan Sulfates

Sanfilippo A (MPS IIIA)	Heparan N-Sulfatase	Heparan Sulfate
Sanfilippo B (MPS IIIB)	α-N-Acetylglucosaminidase	Heparan Sulfate
Sanfilippo C (MPS IIIC)	Acetyl-CoA-Glucosaminide Acetyltransferase	Heparan Sulfate
Sanfilippo D (MPS IIID)	N-Acetylglucosamine –6-Sulfatase	Heparan Sulfate
Morquio A (MPS IVA)	Galactosamine-6-Sulfatase	Keratan Sulfate
Morquio B (MPS IVB)	β-Galactosidase	Keratan Sulfate
Maroteaux-Lamy (MPS VI)	Arylsulfatase B	Dermatan Sulfate
Sly Syndrome (MPS VII)	β-Glucuronidase	
D. Oligosaccharide/Glycoprotein Disorders		
Disease Name	Enzyme Defect	Substance Stored
α–Mannosidosis	α–Mannosidase	Mannose/Oligosaccharides
β-Mannosidosis	β-Mannosidase	Mannose/Oligosaccharides
Fucosidosis	α-L-Fucosidase	Fucosyl Oligosaccharides
Asparylglucosaminuria	N-Aspartyl- β-Glucosaminidase	Asparylglucosamine Asparagines
Sialidosis (Mucolipidosis I)	α-Neuraminidase	Sialyloligosaccharides
Galactosialidosis (Goldberg Syndrome)	Lysosomal Protective Protein Deficiency	Sialyloligosaccharides
Schindler Disease	α-N-Acetyl-Galactosaminidase	
E. Lysosomal Enzyme Transport Disorders		
Disease Name	Enzyme Defect	Substance Stored
Mucolipidosis II (I-Cell Disease)	N-Acetylglucosamine-1- Phosphotransferase	Heparan Sulfate
Mucolipidosis III (Pseudo-Hurler	Same as ML II	

Polydystrophy)		

F. Lysosomal Membrane Transport Disorders		
Disease Name	Enzyme Defect	Substance Stored
Cystinosis	Cystine Transport Protein	Free Cystine
Salla Disease	Sialic Acid Transport Protein	Free Sialic Acid and Glucuronic Acid
Infantile Sialic Acid Storage Disease	Sialic Acid Transport Protein	Free Sialic Acid and Glucuronic Acid

G. Other		
Disease Name	Enzyme Defect	Substance Stored
Batten Disease (Juvenile Neuronal Ceroid Lipofuscinosis)	Unknown	Lipofuscins
Infantile Neuronal Ceroid Lipofuscinosis	Palmitoyl-Protein Thioesterase	Lipofuscins
Mucolipidosis IV	Unknown	Gangliosides & Hyaluronic Acid
Prosaposin	Saposins A, B, C or D	

[0065] In one embodiment of the invention, two or more LSD genes are expressed in a protozoan cell line. In a preferred embodiment, HexA and HexB are coexpressed.

III. Choice of expression system

A. Extrachromosomal or chromosomal expression systems

[0066] According to the invention, a gene product can be expressed from either an extachromosomal (episomal) expression vector, from an expression construct that is integrated into a chromosomal locus, or a combination of both.

[0067] In a preferred embodiment, a stable genomic expression construct is used for *in vivo* delivery. In another embodiment, an episomal expression system is used for in vitro expression. In a further embodiment, a protozoan is engineered to express proteins having a desired carbohydrate structure. A protozoan is engineered to

express one or more glycosylation enzymes in addition to a therapeutic protein of interest. Accordingly, the therapeutic protein is expressed and glycosylated by the recombinant glycosylation enzymes. In a most preferred embodiment, a glycosylation enzyme is used to add mannose-6-phosphate to an expressed protein, thereby providing the expressed protein with a lysosome targeting signal in mammals.

[0068] A non-limiting example of an enzyme expressed in a protozoan is trans-sialidase, which has been expressed in *Leishmania*.

B. Transcriptional and translational control

[0069] In general, high levels of transcription and translation are useful in methods of the invention. According to preferred embodiments of the invention, the coding sequence for an expression product, preferably a lysosomal storage disease gene, is functionally coupled to a regulatory element to obtain expression and secretion of the product (e.g. the lysosomal storage disease protein). According to the invention, useful regulatory elements include promoters, signal peptides, and other elements that are responsible for expression and/or secretion of an expression product such as a lysosomal storage disease protein.

[0070] In a preferred embodiment of the invention, a high level promoter is used to transcribe the gene product of interest.

[0071] In a preferred embodiment of the invention, the sequences surrounding the start codon are optimized for efficient translation in *Leishmania* and/or other protozoa.

[0072] In an alternative embodiment, a gene fragment encoding the structural protein of interest is inserted into an endogenous gene of the protozoan being used. Accordingly, transcription and or translation is achieved using endogenous regulatory sequences.

[0073] In a further embodiment, multiple copies of the gene of interest are introduced into a protozoan, thereby producing a proportional increase in mRNA and protein levels.

C. Use of a signal sequence

[0074] In general, a signal sequence is used to ensure secretion of an expressed gene product according to methods and compositions of the invention.

[0075] In preferred embodiments of the invention, a useful signal peptide is optimized for entry into the secretory pathway of the protozoan that is being used. Example 7 exemplifies an expression construct with a signal peptide that is optimized for *Leishmania*. In general, a signal peptide from an endogenous gene is useful to secrete a heterologous protein from a protozoan. In a preferred embodiment, the coding sequence of a gene of interest is fused, in frame, to a nucleic acid encoding all or part of an endogenous signal peptide. Accordingly, a preferred signal peptide for expression in a chosen protozoan is a signal peptide from that protozoan. However, signal peptides from other organisms, preferably other protozoa, can be used in chimeric expression constructs of the invention.

[0076] Non-limiting examples of *Leishmania* signal peptides include the GP63, secreted acid phosphatase, 3' nucleotidase, chitinase and cystein proteinase signal peptides. Non-limiting examples of *Trypanosoma* signal peptides include the PARP and VSG signal peptides.

[0077] In an alternative embodiment of the invention, the natural signal sequence of the protein being expressed is used. Examples 2-6 show that the natural signal sequences of human GUS and GLA are effective in *L. mexicana*.

[0078] In a further embodiment, a protein is produced in a protozoan that has been modified or selected to increase activity of its secretory pathway.

IV. Administration of expression product to a patient

A. Methods and compositions for administering a gene product purified from an *in vitro* expression system

[0079] Isolated gene products may be administered to a patient using methods known in the art. For example, an LSD protein may be administered to a patient as described in U.S. Patent No. 5,433,946 the disclosure of which is incorporated herein by reference.

B. Methods and compositions for administering a microorganism expressing a gene product

[0080] According to methods of the invention, an engineered protozoan that expresses a gene product is administered to a patient suffering from lack of the gene product.

[0081] In a preferred embodiment, an engineered protozoan is administered intravenously. In alternative embodiments an engineered protozoan is administered subcutaneously, intramuscularly, intraperitonealy, or intracranially. In another embodiment, an engineered protozoan, preferably a gut resident organism, is administered orally. In a further embodiment, an engineered intracellular protozoan is exposed to the patient's cells in vitro. "Infected" cells are then returned to the patient. For example, an engineered *Leishmania* can be used to infect macrophages from a patient in vitro. The infected macrophages are then returned to the patient.

[0082] The amount of an engineered protozoan to be administered to a patient is influenced by several factors, including the expression level of the gene product, the growth characteristics of the protozoan, and the nature of the gene defect in the patient.

[0083] Typically, for lysosomal storage diseases, the amount of enzyme required to rescue the defect is lower than the amount of enzyme present in a healthy individual. Accordingly, a preferred dosage of an engineered protozoan is one that rescues the disease symptoms in the patient.

[0084] Symptoms can be monitored using methods known in the art. The in vivo expression of a gene product according to the invention can be monitored. An example of an in vivo expression assay is shown in Example 9.

[0085] The invention is illustrated further by the following non-limiting examples.

EXAMPLES

Example 1. Method for cloning lysosomal storage disease enzymes into expression vectors.

[0086] The following non-limiting examples illustrate embodiments of the present invention for producing constructs to express lysosomal storage disease enzymes in protozoa. In the following constructs, each LSD gene was cloned into the unique XbaI or BglII sites of the expression plasmid pIR1SAT (FIG. 1A). Nucleic acid fragments containing each LSD gene were generated by PCR amplification of the coding sequence of the LSD gene from a plasmid template obtained from the ATCC, using appropriately designed oligonucleotides having XbaI or BglII restriction sites or sites with overhangs that are complementary to XbaI or BglII restriction overhangs.

i. GLA expression construct

[0087] A human β-galactosidase A (GLA) gene coding fragment was amplified using the following oligonucleotides based on the known GLA sequence:

GLAXX4 5' CGG TCC CCG GGA CAA TGC AGC TGA GGA AC 3'

GLAXXRV 5' ATA TCT AGA TTA AAG TAA GTC TTT TAA TGA 3'

The amplified coding fragment was cloned directionally between the SmaI and XbaI sites of pIR1SAT. The resulting plasmid, pIRGLA-Xa5, contains the GLA insert in the appropriate orientation (in the same orientation as the upstream *L. major* ribosomal small subunit gene (*L. major* SSU in FIG. 1A) so that transcription of the inserted GLA gene is initiated by transcription in the *L. major* ribosomal small subunit gene). The sequence of the GLA insert was verified.

ii. βGUS expression construct

[0088] A human ß-glucuronidase (βGUS) gene coding fragment was amplified using the following oligonucleotides based on the known βGUS sequence:

MULTGUSB4 5' GGT TCT AGA TCT GGG GAC CGG GAA GCA TGG CCC GG 3'

MULTGUSBRV 5' TAT CTA GAT CTT TAG TGT TCC CTG CTA GAA TAG ATG AC 3'

[0089] The amplified coding fragment was cloned into the XbaI site of pIR1SAT. The resulting plasmid, pIRßGUS-X72, contains the βGUS insert in the appropriate orientation (in the same orientation as the upstream *L. major* ribosomal small subunit gene (*L. major* SSU in FIG. 1A) so that transcription of the inserted βGUS gene is initiated by transcription in the *L. major* ribosomal small subunit gene). The sequence of the βGUS insert was verified.

iii. GBA expression construct

[0090] A human glucocerebrosidase (GBA) gene coding fragment was amplified using the following oligonucleotides based on the known GBA sequence:

[0091] DSTGBARV 5' CAC CTC TGC GTG TCG CCC GCA TCG CCT TCC TCT TCC TCT GCC TCA GAT CTA GAC AGT GAG CTC ACC CTA GGC CT 3'

DSTGBA4 5' CCT GTC CCC GGG GGA TCC ACT AGT TCT AGA GGA TCG GAG GTG TGT GAG ATC TCA AGC CTT TGA GTA GGG TAA GC 3'

[0092] The amplified coding fragment was cloned into the XbaI site of pIR1-SAT. The resulting plasmid, pIRGBA-X58, contains the insert in the appropriate orientation (in the same orientation as the upstream *L. major* ribosomal small subunit gene (*L. major* SSU in FIG. 1A) so that transcription of the inserted GBA gene is initiated by transcription in the *L. major* ribosomal small subunit gene). The sequence of the GBA insert was verified.

iv. Other expression constructs

[0093] Using similar amplification and cloning methods, additional clones were produced, including GLA and GBA expression constructs with the coding sequence inserted into the BglII site of pIR1-SAT, Hexosaminidase A (HexA) expression constructs with the coding sequence inserted into the XbaI or the BglII site of pIR1-SAT,

Hexosaminidase B (HexB) expression constructs with the coding sequence inserted into the XbaI or the BglII site of pIR1-SAT, an expression construct with HexA inserted into the BglII site and HexB inserted into the XbaI site of pIR1-SAT, α-Neuraminidase expression constructs with the coding sequence inserted into the XbaI or the BglII site of pIR1-SAT, and N-aspartyl-β-glucosaminidase expression constructs with the coding sequence inserted into the XbaI or the BglII site of pIR1-SAT.

[0094] Similar expression constructs for all LSD enzymes with known sequences can be generated using techniques known in the art.

Example 2. Method for transfecting Leishmania promastigotes

[0095] *Leishmania* promastigotes were maintained in culture at 26-27°C by serial passage of 10 mL cultures at dilutions of 1:100 in 25cm^3 tissue culture flasks containing complete M199 media described in LeBowitz, J. H. (1994, Transfection experiments with *Leishmania*. Microbes as tools for cell biology. D. G. Russell. San Diego, Academic Press. 45: 65-78). Under these conditions, doubling times for promastigote cultures range from about 8-20 hours and saturation densities range from 1-8 x10^7 cells/mL depending on the particular species and isolate.

[0096] Promastiogotes were transfected as follows. Promastigote preparations were electroporated with either 1 μg of gel purified linear expression construct DNA for genomic integration and expression or with 10 μg plasmid DNA for episomal expression. Selection of Leishmania clonal transfectants was carried out as described in LeBowitz, J. H. (1994, Transfection experiments with *Leishmania*. Microbes as tools for cell biology. D. G. Russell. San Diego, Academic Press. 45: 65-78. The selective antibiotic, nourseothricin was present in selective plates at a concentration of 100 μg/mL and in liquid cultures at 50 μg/mL.

[0097] In order to increase the number of chromosomal inserts, 10 μg of linear DNA may be electroporated into a promastigote preparation in order to induce the integration of multiple gene copies.

Example 3. Methods for assaying lysosomal storage disease enzyme activities in promastigotes

[0098] In the following non-limiting examples, LSD enzymes were assayed in both culture media and cell lysates to monitor the extent of enzyme secretion. An assay for glucose-6-phosphate dehydrogenase (G6PDH), a cytosolic protein, was used as a control for cell partitioning. Samples were performed in triplicate.

i. GLA assay

[0099] GLA levels were measured using the assay described below, adapted from Desnick et al., J. Lab. And Clin. Medicine (1973) 81:157-171.

ii. GBA assay

[0100] GBA levels were measured using the assay described below.

iii. βGUS assay

[0101] βGUS levels were measured using the assay described below, adapted from Wolfe and Sands, Protocols for Gene Transfer in Neuroscience (1996), Ed. Lowenstein and Enquist, Chp. 20.

iv. G6PDH assay

[0102] G6PDH levels were measured using the assay described below, adapted from Heise and Opperdoes, MBP 99(1999) 21-32, and Leishmania Lysates: LeBowitz, Trends Cell Biol, (1994) 45 p.63-76.

v. Assay reagents

[0103] The stock solutions used in the assays are as follows. 100X GLA Substrate is 5 mL of absolute EtOH added to a 250 mg vial of 4-methylumbelliferyl β-D-GAL (4-MU-β-D GAL) (the substrate does not dissolve, and it is stored in a vial in a –20°C dessicator with the cap wrapped in parafilm). 100X GBA Substrate is 690 mg 4-MU-β-D glucopyranoside (glucoside) (MUBGlc) added to 8 mL absolute EtOH (again

the substrate does not dissolve, and it is stored in a vial in a –20°C dessicator with the cap wrapped in parafilm). 25X GUS Substrate is 4.55 mL absolute ethanol (EtOH) added to a 250 mg vial of 4-MU-β-D glucuronide (MUG) (again the substrate does not dissolve, and it is stored in a vial in a –20°C dessicator with the cap wrapped in parafilm). 100mM triethanolamine, pH 8 is 9.3 g triethanolamine (Sigma T-9534) dissolved in 500 mL water, adjusted to pH 8, and filter-sterilized. 100X $NADP^+$ (50mM) is 766 mg $NADP^+$ (Sigma N-0505) dissolved in 20 mL 100mM triethanolamine, pH 8, and filter-sterilized. 100X D-glucose 6-phosphate (G6P) (500mM) is 2.82 g G6P (Sigma G-7879) dissolved in 20 mL 100mM triethanolamine, pH 8, and filter-sterilized. 10X Cit/Phos Buffer (0.5M citric acid, 1M Na_2PO_4, pH 4.7) is 10.5 g citric acid and 26.8 g Na_2PO_4 dissolved in 67 mL water, pH to 4.7, filled to 100 mL, and filter-sterilized. Other stock solutions used are 1M Sodium Acetate, pH 4.8 (pH with acetic acid), Triton X-100, Hank's Balanced Salt Solution (HBSS) (Gibco), and 1M $MgCl_2$. The protease inhibitor stock solutions (See LeBowitz Trends Cell Biol, (1994) 45 p.63-76) are 100 mg/mL benzamidine in EtOH, 5 mg/mL leupeptin, 100 mg/mL 1,10 phenanthroline, 0.5 M EDTA, 10 mg/mL soybean trypsin inhibitor, 10 mg/mL BSA (molecular biology grade), and 100 mg/mL PMSF in methanol.

[0104] The working solutions used in the assays are as follows. Cell Lysis Mix is made fresh with the following components (per 1 mL) 980 µL of HBSS, 1.5 µL of Benzamidine, 4 µL of Leupeptin, 2 µL of Phenanthroline, 2 µL of 0.5 M EDTA, 5 µL Trypsin inhibitor, 5 µL of BSA, 1.7 µL of PMSF, and 10 µL of Triton X-100. The Stop Solution is 10.6 g Na_2CO_3, 12.01 g Glycine, H_2O to 500 mL, with the pH adjusted to 10.5, and filter-sterilized. GLA reaction mix is (per 1mL) 890 µL H_2O, 100 µL 10X cit/phos buffer, and 10 µL 100X GLA substrate. GBA reaction mix is (per 1mL) 890 µL H_2O, 100 µL 10X cit/phos buffer, and 10 µL 100X GBA substrate. GUS reaction mix is (per 1mL) 860 µL H_2O, 100 µL 1M NaAc, and 40 µL 25X GUS substrate. G6PDH reaction mix is (per 100mL), 98 mL 100 mM triethanol amine pH 8, 50 µL 1 M $MgCl_2$, 1 mL 100X □□$NADP^+$, and 1 mL 100X G6P.

vi. Assay protocol

[0105] Assays to measure levels of GLA, GBA, GUS and G6PDH were performed using the following protocol.

1) Number 1.5 mL eppendorf tubes, Coulter cups, spectrophotometer and fluoremeter cuvettes (both types of cuvettes should be labeled in tripticate). Fill Coulter cups with 10 mL electrolyte solution.
2) Transfer 1 mL *Leishmania* cells into eppendorf tubes and 0.1 mL cells into Coulter cups containing electrolyte solution. Obtain cell counts when time is available (during later incubation steps). Note: samples do not have to be in triplicate at this point (see steps 6-7)
3) Harvest cells in eppendorf tubes at 3K rpm for 5 min. Remove ~500 mL media (without disturbing cell pellet) from each tube and transfer to a new eppendorf tube. Aspirate off remaining media.
4) Wash each cell pellet in 500 mL HBSS. Spin as above, aspirate HBSS, and suspend cell pellets in 100 μL lysis buffer.
5) Pulse-vortex cells and incubate on ice for 15 minutes. Spin lysates at full-speed for 15 min to pellet cell debris.
6) Transfer 150 μL of the appropriate reaction mix (GLA, GBA or GUS) to fluorimeter cuvettes (in triplicate) and 1 mL of the G6PDH reaction mix to spectrophotometer cuvettes (in triplicate).
7) Transfer 38 μL of media into GLA, GBA or GUS cuvettes and G6PDH cuvettes. Transfer 3.8 μL of cell lysates into GLA,GBA or GUS cuvettes and G6PDH cuvettes.
8) Cover GLA and GUS cuvettes with parafilm (do not cover cuvette box with lid) and incubate 1 hour in 37°C incubator. Incubate G6PDH cuvettes at room temperature overnight.
9) Stop GLA, GBA and GUS reactions by adding 2 mL of glycine/carbonate stop solution.
10) Read fluorescence on Versafluor fluorimeter.

vii. Results

[0106] Expression of ßGUS and GLA was shown in both *L. mexicana* and in *L. major* Δ*DHFR-TS*).

[0107] FIGs 2 and 3 show that GLA is secreted from *L. mexicana* promastigotes into the culture medium. FIGs 4 and 5 show that ßGUS is secreted from *L. mexicana* promastigotes into the culture medium. FIGs 8 and 9 show that GLA and ßGUS were expressed from many independently isolated *L. mexicana* cell lines. FIGs 10A and 10B show that GLA and ßGUS accumulate in *L. mexicana* culture supernatants over time.

[0108] Expression of GBA, HexA, HexB, and HexS was also shown in *Leishmania* as described in Example 12. Other examples of preferred constructs include neuraminidase (NEU1) and aspartylglucosaminidase (AGU) expression constructs.

Example 4. Methods for assaying lysosomal storage disease enzyme activities in amastigotes

[0109] Promastigotes of *L. mexicana* were converted to amastigotes by pelleting ~ 0.5 mL of promastigote culture, removing the M199 media, then resuspending the cells in 5 mL of modified UM54 medium (Moore, Santrich et al. 1996). Flasks were incubated horizontally at 32-33°C. Amastigotes are passed by 1:10 dilution into pre-warmed UM54 every 3-4 days.

[0110] Assays for LSD enzyme expression were performed on amastigote cultures derived from the promastigote cell lines described above. FIGs 6 and 7 show that GLA and ßGUS are secreted from *L. mexicana* amastigotes into the culture medium.

Example 5. Gene Product Expression in serum free media

[0111] *Leishmania tarentolae* expressing a lysosomal storage disease enzyme is grown in medium in the absence of serum in HyQ™ FXS Serum Free X-Insect (HyClone). The culture is grown to high density, and the enzyme is purified from the culture supernatant.

[0112] Expression product can also be isolated from serum free media using other protozoa, including other *Leishmania* species. In general, the expression strain is grown in medium with serum, diluted into serum free medium, and allowed to grow for several generations, preferably 2-5 generations, before the expression product is isolated. For example, production of secreted recombinant LSD proteins can be isolated from *Leishmania mexicana* promastigotes that are cultured initially in 50 mL 1X M199 medium in a 75 cm2 flask at 27° C. When the cell density reaches 1-3x 10^7/mL, the culture is used to inoculate 1.2 L of M199 media. When the density of this culture reaches about 5x10^6/mL, the cells were harvested by centrifugation, resuspended in 180 mL of the supernatant and used to inoculate 12 L of "Zima" medium in a 16 L spinner flask. The initial cell density of this culture is typically about 5x 10^5 /mL. This culture is expanded to a cell density of about 1.0 – 1.7 x 10e^7 cells/mL. When this cell density is reached, the cells are separated from the culture medium by centrifugation and the supernatant is filtered at 4oC through a 0.2 μ filter to remove residual promastigotes. The filtered media was concentrated from 12.0 L to 500 mL using a tangential flow filtration device (MILLIPORE Prep/Scale-TFF cartridge).

[0113] Preferred growth media for this method are M199 and "Zima" growth media. However, other serum containing and serum free media are also useful. M199 growth media is as follows: (1L batch) = 200 mL 5X M199 (with phenol pH indicator) mixed at 5X + 637 mL H_2O, 50.0 mL FBS, 50.0 mL EF, 20.0 mL of 50g/mL SAT, 2.0 mL of 0.25% hemin in 50% triethanolamine , 10 mL of 10mM adenine in 50mM Hepes pH 7.5, 40.0 mL of 1M Hepes pH 7.5, 1mL of 0.1% biotin in 95% ethanol, 10.0 mL of penicillin/streptomycin. All serums used are inactivated by heat. The final volume = 1 L and is filter sterilized. "Zima" modified M199 media is as follows: (20.0 L batch) = 217.8g M199 powder (–)phenol red + 7.0g sodium bicarbonate, 200.0 mL of 10mM adenine in 50mM Hepes pH 7.5, 800.0 mL 0f Hepes free acid pH 7.5, 20.0 mL 0.1% biotin in 95% ethanol, 200.0 mL penicillin/streptomycin, 2780.0 mL H20 Final volume = 20.0 L and is filter sterilized.

Example 6. *Leishmania* expression vectors

[0114] The following non-limiting example is illustrative of methods and compositions for increasing expression and secretion of gene products in protozoa. Useful secretion of an LSD enzyme from *Leishmania* will be obtained using an expression vector that facilitates the construction and expression of hybrid proteins containing a signal peptide derived from a *Leishmania* protein, fused to the portion of the LSD gene that encodes the mature LSD enzyme. Useful expression will be obtained using a signal peptide that is optimized for maximal expression. Useful expression will also be obtained using a vector in which sequences 5' to the start codon are optimized for maximal translation. Translation of a gene coding for an LSD enzyme will be maximized in *Leishmania* by altering the codon usage of the LSD gene to reflect the codon bias of *Leishmania*. Useful expression of LSD genes will be driven by including a bacteriophage RNA polymerases (such as T-7 RNA polymerase) in an expression vector. Examples of different expression systems for optimizing gene, and particularly LSD gene expression and/or secretion are provided in Example 13.

Example 7. Signal Peptide Increased Translation and Secretion Plasmid

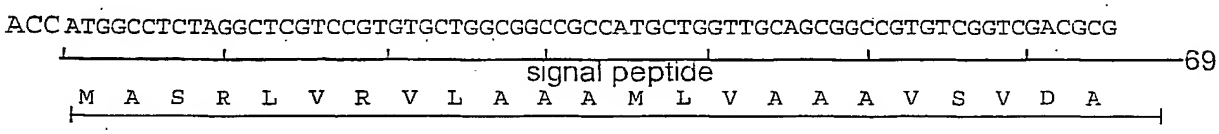
[0115] Plasmid pSPITS (Signal Peptide Increased Translation & Secretion) is shown in FIG. 1B. Sequences between the unique NarI and XbaI sites of pIR1-SAT are replaced with sequences from the *L. major* gp63 locus containing the 5' intergenic region, including a putative splice acceptor site and the 5' end of the polypeptide containing the signal peptide. This cassette is amplified from *L. major* genomic DNA using oligos:

GP63 5' 5' CAGATCGATCTCGAGGGTGCTGTCCCCCCTCGCTGCGGCGT 3'
GP63 3' 5' AGGTCTAGATGCCCACGCGGCCGCGGTGCCGACA 3'

[0116] The 5' oligo has a ClaI site that is compatible with the NarI site in the vector and the 3' oligo has an XbaI site. Gene cassettes are easily cloned into the resulting plasmid, between the unique NotI and XbaI sites. Appropriately designed oligonucleotides are used to amplify a gene cassette by PCR, the gene cassette is cloned

into pSPITS and the resulting plasmid expresses a chimeric gene containing the gp63 signal peptide fused to the polypeptide encoded by the gene cassette.

[0117] An alternative construct is made by inserting an oligonucleotide encoding the signal peptide of the *L. mexicana* secreted acid phosphatase, SAP-1, into the XbaI site of a modified pIR1-SAT in which the single SalI site has been removed. Appropriately designed oligonucleotides are used to amplify a gene cassette by PCR, and the gene cassette is cloned into the plasmid. The resulting plasmid expresses a chimeric gene containing the SAP signal peptide fused to the polypeptide encoded by the gene cassette.



SAP-1 signal peptide.

[0118] To conform the 3 nucleotides 5' to the start codon of ßGUS and GLA to desirable sequences for optimal translation in *Leishmania* expression constructs were made using the following oligonucleotides to modify the 5' region. In each case, the relevant oligonucleotides used to amplify the gene cassettes are listed below with the altered trinucleotide sequence highlighted in bold. The first oligonucleotide of each set has the endogenous sequence.

Oligonucleotides for GLA 5' modification:

GLAXACA 5' CCACTCTAGAACAATGCAGCTGAGGAACCCAGAACTA 37

GLAXACC 5' CCACTCTAGA**ACC**ATGCAGCTGAGGAACCCAGAACTA 37

GLAXATC 5' CCACTCTAGA**ATC**ATGCAGCTGAGGAACCCAGAACTA 37

GLAXCCC 5' CCACTCTAGA**CCC**ATGCAGCTGAGGAACCCAGAACTA 37

Oligonucleotides for GUS 5' modification:

GUSXAGC 5' CCACTCTAGAAGCATGGCCCGGGGGTCGGCGGTTGCC 37

GUSXACC 5' CCACTCTAGAACCATGGCCCGGGGGTCGGCGGTTGCC 37

GUSXATC 5' CCACTCTAGAATCATGGCCCGGGGGTCGGCGGTTGCC 37

Example 8. Vectors for tagging expression products

[0119] The following non-limiting example illustrates the modification of a protozoan expression system to tag the expressed gene product.

[0120] Modified pIR1-SAT vectors designed to create a C-terminal epitope tagged expression product were constructed. The constructs have a VSV tag (mab available from Roche) inserted into either the XbaI site or the BglII site of pIR1SAT.

[0121] Oligonucleotides for making the tag constructs are VSVB1 and VSVB2 for the BglII site, and VSVX1 and VSVX2 for the XbaI site. For both modifications there are unique XhoI and Asp718 sites that permit appropriately amplified gene cassettes to be ligated in frame with the tag cassette.

[0122] Gla and Gus cassettes were cloned into the XbaI tag vector using each of the 5' modified nucleotide sequences.

Oligonucleotides for the BglII tag site:

GATCTCGGGTACCGCTCGAGTACACGGATATCGAGATGAACCGCCTGGGCAA GTGATCGAT VSVB1

GATCATCGATCACTTGCCCAGGCGGTTCATCTCGATATCCGTGTACTCGAGCG GTACCCGA VSVB2

Oligonucleotides for the XbaI tag site:

CTAGACGGGTACCGCTCGAGTACACGGATATCGAGATGAACCGCCTGGGCAA GTGATCGATA VSVX1

CTAGTATCGATCACTTGCCCAGGCGGTTCATCTCGATATCCGTGTACTCGAGC
GGTACCCGT VSVX2

Example 9. *In vivo* expression assays

[0123] *In vivo* expression of an LSD protein is assayed using an enzyme-based, a histochemical, or an immunological method, or a combination of the above, according to methods known in the art.

[0124] According to the invention, to demonstrate that LSD proteins secreted from *Leishmania* contain carbohydrate with terminal mannose residues, recombinant ß-glucuronidase from *Leishmania mexicana* containing plasmsid pXSAP0-GUS was grown in M199 culture medium with a small amount of serum proteins. When the culture reached a density of > 1.0×10^7 promastigotes/mL the *L. mexicana* were removed by centrifugation, 10 min at 500 x g. The harvested culture medium was passed through a 0.2 µm filter to remove particulates before being loaded directly onto a Concanavalin A (ConA)-agarose column (4% cross-linked beaded agarose, Sigma). The ConA-agarose column was pretreated with 1 M NaCl, 20 mM Tris pH 7.4, 5 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$ and then equilibrated with 5 volumes of column buffer (20 mM Tris pH 7.4, 1 mM $CaCl_2$, and 1 mM $MnCl_2$). A total of 179,800 units (nmol/hr) of GUS activity (in 2 L) in culture medium was loaded onto a 22 mL ConA agarose column. No activity was detectable in the flow through or wash. The GUS activity was eluted with column buffer containing 200 mM methyl mannopyranoside. Eluted fractions containing the activity peak were pooled and concentrated: 143900 units of GUS activity were recovered from the column (80% recovery of activity loaded onto the column). This demonstrates that the recombinant ß-GUS secreted from *L. mexicana* did possess carbohydrate with terminal mannose residues and further points out the potential for using the interaction of mannose with ConA as the basis for an affinity purification step.the presence of high mannose carbohydrate can serve as the basis of an affinity step in the purification of recombinant LSD proteins using lectin affinity chromatography.

Example 10. Mannose or mannose-6-phosphate Mediated Internalization of lysosomal Storage Disease Proteins

[0125] In order for LSD proteins produced by stably transfected protozoa to be an effective therapeutic agent for lysosomal storage diseases, the enzyme must be internalized by the affected cells and transported to the lysosome. In most cases, this internalization is mediated by the binding of lysosomal enzymes to mannose-6-phosphate (M6P) receptors, which are expressed on the cell surface of most cell types and deliver the enzyme to the lysosome via the endocytic pathway. The M6P receptor is ubiquitously expressed; most somatic cells express it to some extent. The mannose receptor, which is specific for exposed mannose residues on glycoproteins, is less prevalent. The latter receptors are generally found only on macrophage and macrophage-like cells. The mannose receptor provides an essential means of entry for glucocerebrosidase into cells of the macrophage lineage where it can exert a therapeutic effect.

[0126] In order to demonstrate M6P-mediated internalization of lysosomal enzymes, skin fibroblasts from disease patients (NIGMS Human Genetic Mutant Cell Repository) will be cultured overnight in the presence of increasing concentrations of purified .enzyme of the invention. Some of the samples will contain 5 mM soluble M6P, which competitively inhibits binding to, and as a result, internalization by, the mannose-6 phosphate receptor. Other samples will contain 30 .µg/mL mannan, which inhibits binding to, and as a result, internalization by, the mannose receptor. Following incubation, the cells will be washed and harvested by scraping into lysis buffer (10 mM Tris, pH 7.2, 100 mM NaCl, 5 mM EDTA, 2 mM Pefabloc.TM. (Roche) and 1% NP-40). The lysed samples will then be assayed for protein concentration and enzyme activity. The results will be expressed as units of enzyme activity/mg cell protein. It is expected that enzymes internalized via the M6P receptor will display a significant amount of cell associated activity that can be competed by the addition of M6P to the media but not by the addition of mannan.

[0127] The mouse macrophage-like cell line (J774.E) which bears mannose receptors but few if any mannose 6-phosphate receptors can be used to determine whether purified glucocerebrosidase of the invention is internalized via the

mannose receptor (Diment et al., J. Leukocyte Biol.42:485-490, 1987). J774.E cells will be cultured overnight in the presence of increasing concentrations of glucocerebrosidase. Selected samples will contain 2 mM M6P, and others will contain 100 µg/mL mannan. The cells will be washed and harvested as described above, and the total protein and glucocerebrosidase activity of each sample will be determined. It is predicted that M6P will not inhibit the uptake of glucocerebrosidase by these cells, while mannan decreases the accumulated glucocerebrosidase significantly.

Example 11. Mannose Mediated Internalization of Glucocerebrosidase

[0128] In order for glucocerebrosidase produced by stably transfected protozoa to be an effective therapeutic agent for Gaucher's disease, the enzyme must be internalized by cells of the macrophage lineage and transported to the lysosome. This internalization is mediated by the binding of glucocerebrosidase to the mannose receptor, which is specific for exposed mannose residues on glycoproteins. The mannose receptors are generally found only on macrophage and macrophage-like cells. The mannose receptor provides a means of entry for glucocerebrosidase into cells of the macrophage lineage where they can exert a therapeutic effect.

[0129] The mouse macrophage-like cell line (J774.E) which bears mannose receptors but few if any mannose 6-phosphate receptors can be used to determine whether purified glucocerebrosidase of the invention is internalized via the mannose receptor (Diment et al., J. Leukocyte Biol.42:485-490, 1987). J774.E cells will be cultured overnight in the presence of increasing concentrations of glucocerebrosidase. Some of the samples will will contain 2 mM M6P, which competitively inhibits binding to, and as a result, internalization by, the mannose-6 phosphate receptor. Other samples will contain 100 µg/mL mannan, which inhibits binding to, and as a result, internalization by, the mannose receptor. Following incubation, the cells will be washed and harvested by scraping into lysis buffer (10 mM Tris, pH 7.2, 100 mM NaCl, 5 mM EDTA, 2 mM Pefabloc.TM. (Roche) and 1% NP-40). The lysed samples will then be assayed for protein concentration and glucocerebrosidase activity. The results will be expressed as units of glucocerebrosidase activity/mg cell protein. It is expected that

glucocerebrosidase internalized via the mannose receptor will display a significant amount of cell associated activity that can be competed by the addition of mannan to the media but not by the addition of M6P.

Example 12. Expression and Secretion of GBA and HexA, HexB, and HexS

[0130] *Leishmania* strains have been engineered to secrete active glucocerebrosidase (GBA) and hexosaminidaseS (HexS), hexosaminidaseB (HexB) and hexosaminidaseA (HexA). Each of these enzymes are secreted in both the promastigote and amastigote stages.

i. GBA Expression

[0131] GBA was successfully expressed using either of two *Leishmania* signal peptides and have also using the longer of two putative naturally occurring GBA signal peptides. Figures 11 and 12 show GBA expression from SAP-GBA constructs described below.

ii. HexA, HexB, and HexS Expression

[0132] HexS protein is a homodimeric protein where each subunit is encoded by the HexA gene. HexB is a homodimeric protein where each subunit is encoded by the HexB gene. HexA protein is a heterodimeric protein where one subunit is encoded by the HexA gene and the other subunit is encoded by the HexB gene. Mutations in the HexA gene are responsible for Tay Sachs disease, but it the pathology is reportedly due to the absence of functional HexA protein and not HexS protein. Sandhoff disease is caused by mutations in the HexB gene, and the pathology is due to lack of functional HexA and HexB. Accordingly, there is a need for methods for expressing, isolating, and delivering functional hexosaminidase enzymes.

[0133] Expression cassettes were made in which the HexA or HexB genes were separately expressed. In addition, constructs were made to simultaneously coexpress the HexA and HexB genes. Two substrates were used to assay the Hex

proteins (see Figures 13 and 14). MUG (4-methylumbelliferyl-GlcNAc) is hydrolyzed by HexA, HexB and HexS proteins. MUGS (4-methylumbelliferyl-GlcNAc-6-SO_4) is hydrolyzed by HexA and HexS only.

Example 13. Expression and Secretion Systems

[0134] To illustrate the general applicability of the invention, three alternative vectors were made to permit fusion of different *Leishmania* signal peptides with mature LSD polypeptides. The first vector uses the signal peptide from SAP (secreted acid phosphatase) and was constructed as described herein. The other two vectors designated pSPITS-1 (referred to as pSPITS in Example 1) and pSPITS-3 contain the gp63 signal peptide and either about 280 or 460 bp of 5' flanking sequence from gp63 cloned into pIR1-SAT as previously herein.

[0135] Each of the GLA, ß-GUS, and GBA cassettes have been inserted into the signal peptide vectors in three ways creating chimeric genes in which the mature gene cassette is fused either at the site of signal peptide cleavage, 1 amino acid residue downstream from the site of cleavage, or 5 amino acids downstream from the site of cleavage. In the latter two cases, the resultant chimeric LSD proteins would have 1 or 5 amino acids at the amino terminus encoded by the *Leishmania* gene that provided the signal peptide.

[0136] Exemplary expression strains described herein follow the following nomenclature. *Leishmania* strains are named with a number and further by denoting the plasmid used for transfection. All plasmids are derived from pIR1-SAT. Plasmids with gene cassettes encoding ß-Glucuronidase contain the name GUS; those with glucocerebrosidase contain GBA in the name; those with a-Galactosidase A contain GLA in the name. Vectors in which the *Leishmania* secreted acid phosphatase signal peptide is fused to the mature LSD gene cassette contain SAP in the name; vectors using the gp63 signal peptide and intergenic region have either pSPITS1 or pSPITS3 in the name depending on the size of the gp63 intergenic region employed. SAP and pSPITS constructs have a further number designation that indicates the site of fusion of the signal

peptide with the LSD gene. For example, in the name pSPITS3-GUS0-93 the '0' after GUS refers to the fact that the fusion is at position 0 relative to the gp63 cleavage site. In pSPITS3-GUS5-100, the '5' refers to the fact that 5 residues downstream of the gp63 cleavage site were included in the signal peptide cassette fused to mature GUS. The same applies to SAP constructs except that the number can occur either after the SAP or after the gene name. For example in the names pSAP-GUS0-21 and pGLA-SAP0-1, the '0' in each case refers to the fusion of the signal peptide to the LSD cassette at position 0 relative to the cleavage site of the SAP signal peptide. Constructs with an 'X' in the name, refer to the cassette being inserted between the XmaI and XbaI sites in the vector.

[0137] Preferred expression constructs are based on combinations of regulatory elements disclosed herein. Preferred expression construct combinations for GBA include those on the following plasmids: pGBA-SAP0, pGBA-SAP1, pGBA-SAP5, pSPITS1-GBA0, pSPITS1-GBA1, pSPITS3-GBA0, pSPITS3-GBA1, pSPITS3-GBA5, pSAP0-GBA, pXGBA and pXGBA-SAP0. Preferred expression construct combinations for GUS include those on the following plasmids: pSAP-GUS0, pSAP-GUS1, pSAP-GUS5, pSPITS1-GUS0, pSPITS1-GUS1, pSPITS3-GUS0, pSPITS3-GUS1, pSPITS3-GUS5, and pXGUS-SAP0. Preferred expression construct combinations for GLA include those on the following plasmids: pGLA-SAP0, pGLA-SAP1, pGLA-SAP5, pSPITS1-GLA0, and pSPITS3-GLA0. These and other combinations of regulatory elements disclosed herein are useful for the expression and secretion of other LSD enzymes in addition to GBA, GUS, and GLA. Alternative expression construct combinations include pGLA-CCC-XTAG, pGLA-ATC-XTAG, pIR-GLA-CCC, pIR-GLABw, pIR-GLA-Xd, pGLA-ACA-XTAG, pGLA-ACC-XTAG, and pIR-ACC-GLA. Different plasmid, regulatory element and gene combinations produced different levels of expression and secretion. For example, the GBA-SAP1 construct gave the highest activity while the SAP-0 construct was less active. However, there is a point mutation in the GBA-SAP -0 construct that changes a threonine residue to an alanine residue, and this could alter the activity of the protein. In another example, higher levels of ß-glucuronidase and α galactosidase A expression in *Leishmania* were achieved with constructs using the *Leishmania* secreted acid phosphatase (SAP) signal peptide. In another example, recombinant *Leishmania* containing gene cassettes in which the

nucleotides upstream of the start codon of ß-GUS, GLA and GBA have been altered. Some of these alterations reproducibly increase expression levels. As a further example, relative ß-GUS activities with different constructs were also tested. In another example, *Leishmania* that express epitope-tagged versions of GLA, ß-GUS and GBA were tested and displayed activity, and tagged proteins (e.g. the tagged- ß-GUS protein) can be detected by western blot.

[0138] The following tables show expression levels for examples of different expression constructs.

Table 3. Relative ß-GUS activities in promastigotes and amastigotes containing different GUS constructs. The Xba and Bgl constructs refer to the original constructs in which the ß-GUS cassette was cloned into the XbaI or BglII site of pIR1-SAT. ACC, ATC etc refer to the constructs in which the nucleotides 5' of the start codon were altered. XTAG refers to the presence of a carboxy terminal VSV- epitope tag.

	Normalized expression			
	Promastigotes		Amastigotes	
Construct	Media	Cells	Media	Cells
Xba	1.00	0.77	1.51	1.66
Bgl	0.58	0.31	0.92	1.02
ACC	3.58	2.29	4.18	2.10
ATC	2.15	1.80	5.05	3.27
AGC	2.71	1.68	3.58	1.99
ACC-XTAG	0.45	3.34	1.00	5.42
ATC-XTAG	0.78	4.31	0.69	3.86
AGC-XTAG	0.54	3.34	0.82	4.58

The following tables show exemplary expression levels in different *L. mexicana* strains.

Table 4: Examples of ecreted activity from *L. mexicana* promastigotes.

Gene	Strain	U in media/ 10^6 promastigotes	U in media/ 10^6 amastigotes
ß-glucuronidase	499.4 pXGUS-SAP0-2	12.31	NA
Glucocerebrosidase	449.4 pSPITS1-GBA1-53	0.96	4.43
α-Galactosidase A	383.1 pGLA-SAP0-6	2.94	0.56

Table 5: Expression of glucocerebrosidase in *L. mexicana* using different signal peptides.

Signal peptide	Strain	U in media/ 10^6 promastigotes	U in media/ 10^6 amastigotes
Endogenous short	167.1 pIRSATGBAX58	0	0
Endogenous long	513.1 pXGBA-25	0.29	NA
SAP	511.3 pXGBA-SAP0-21	0.38	NA
gp63	445.4 pSPITS1-GBA0-49	1.02	3.72

[0139] In addition, several ß-GUS constructs were tested in the ΔDHFR-TS strain of *L. major Freidlen*. Activities approximately paralleled those observed in *L. mexicana*, as shown in the following table.

Table 6: Expression of selected constructs in *L. mexicana* and *L. major Freidlen* Δdhfr-ts.

Plasmid	*Leishmania Mexicana* strain	U in media/ 10^6 cells	*Δdhfr-ts Leishmania major Freidlen* strain	U in media/ 10^6 cells
PXGUS-SAP0-1	497.3	11.6	534.5	9.21
PSAP-GUS1-25	409.5	6.43	537.2	10.67
PSPITS3-GUS0-93	488.2	2.98	539.5	4.40

CLAIMS

What is claimed is:

1. A method for obtaining a lysosomal storage disease enzyme, comprising:

(a) providing a protozoan that expresses a lysosomal storage disease enzyme;

(b) growing said protozoan in an *in vitro* culture; and,

(c) purifying said enzyme from said culture.

2. A method for delivering a lysosomal storage disease enzyme to a patient, comprising:

(a) providing a protozoan that expresses a lysosomal storage disease enzyme under the control of a regulatory element that drives expression and secretion of said enzyme; and,

(b) administering said protozoan to a patient,

wherein said protozoan persists in said host and delivers said enzyme to said patient.

3. A protozoan engineered to express a lysosomal storage disease enzyme, comprising:

(a) a lysosomal storage disease enzyme coding sequence; and,

(b) a regulatory element driving gene expression functionally coupled to said coding sequence,

wherein transcription from said promoter expresses said lysosomal storage disease enzyme.

4. A method for obtaining a prion free expression protein preparation, comprising

(a) providing a protozoan that is engineered to express a protein;

(b) growing said engineered protozoan in a culture medium that is free of factors derived from a mammalian source; and,

(c) purifying said protein from said protozoan culture.

5. The method of claims 2 or 3, wherein said regulatory element comprises a signal peptide from said protozoan.

6. The method of claim 5, wherein said signal peptide is selected from the group consisting of SAP and gp63 signal peptides.

7. The method of claims 1, 2, or 4, wherein said protozoan is a trypanosomatid.

8. The method of claim 7, wherein said trypanosomatid is a *Leishmania* species.

9. The method of claim 8, wherein said *Leishmania* species is selected from the group consisting of *L. mexicana*, *L. major*, and *L. tarentolae*.

10. The protozoan of claim 3, wherein said protozoan is a trypanosomatid.

11. The protozoan of claim 10, wherein said trypanosomatid is a *Leishmania* species.

12. The protozoan of claim 11, wherein said *Leishmania* species is selected from the group consisting of *L. mexicana*, *L. major*, and *L. tarentolae*.

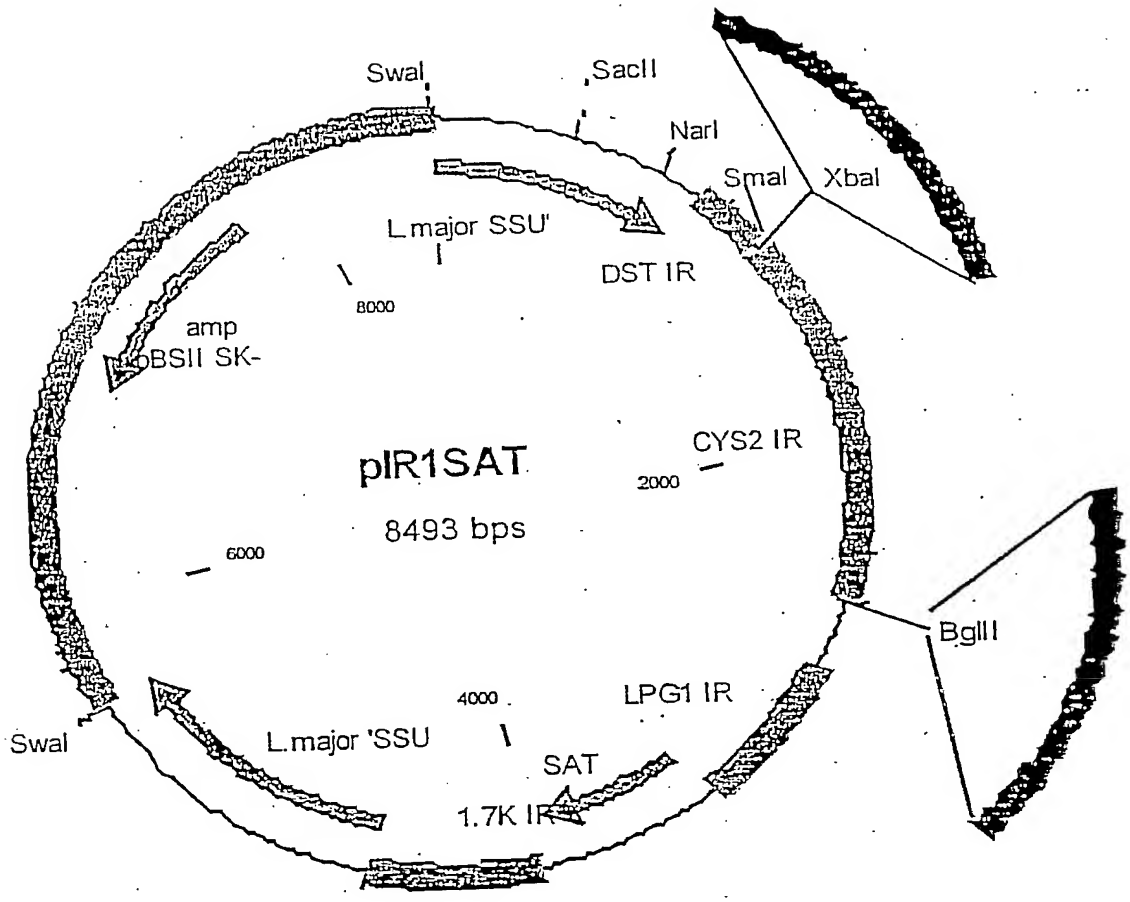
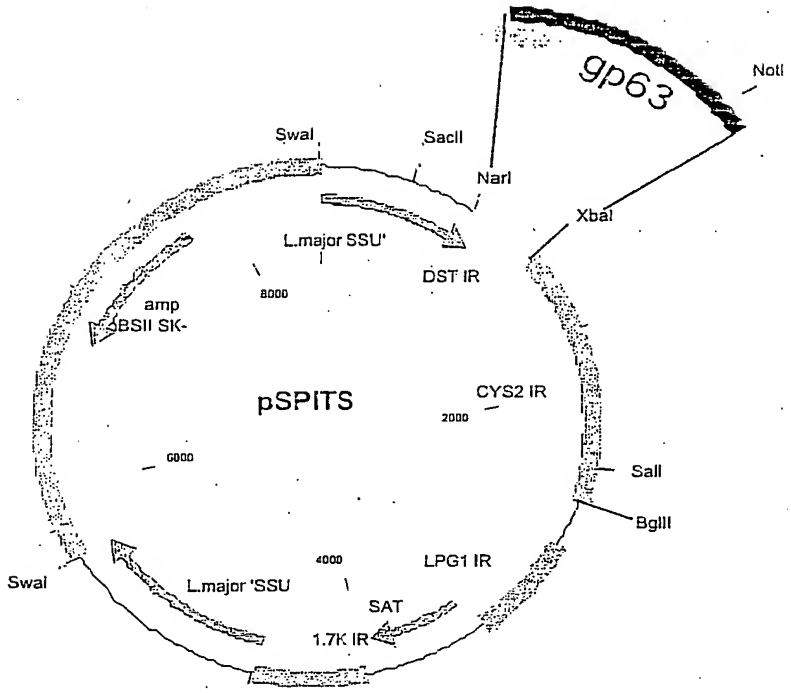


Figure 1A



GAGTGGCGGGGTGCTGTCCCCCCTCGCTGCGGCGTCCCTCCCCGCGCCAGCACGCACGCACTCCCGTGCCCGTGA

75

CCCCCTCCGCACCAGCACCGATGCGCACTCCGCTCTCCCTCCCCTCGCACCCTCCCTCGCCCATCCCTTGCCGCC

150

TCCCTGTCCCCTCCCTCCCCAGATCCGCCAACGCATCCGATCCCGCTACACCCTCTCCCCCGCCCATACGCACGC

225

GCACACCGCCGTGCACAAGCCCTCGCCCTCGCCACCACACCCCACTGCCCACAGCGCCCCCGCGCCTGCAGAGCC

300

ATGTCCGTGGACAGCAGCAGCACGCACCGGCGCCGCTGCGTCGCCGCGCGCCTGGTGCGCCTCGCGGCTGCCGGC

375

Signal Peptide

M S V D S S S T H R R R C V A A R L V R L A A A G

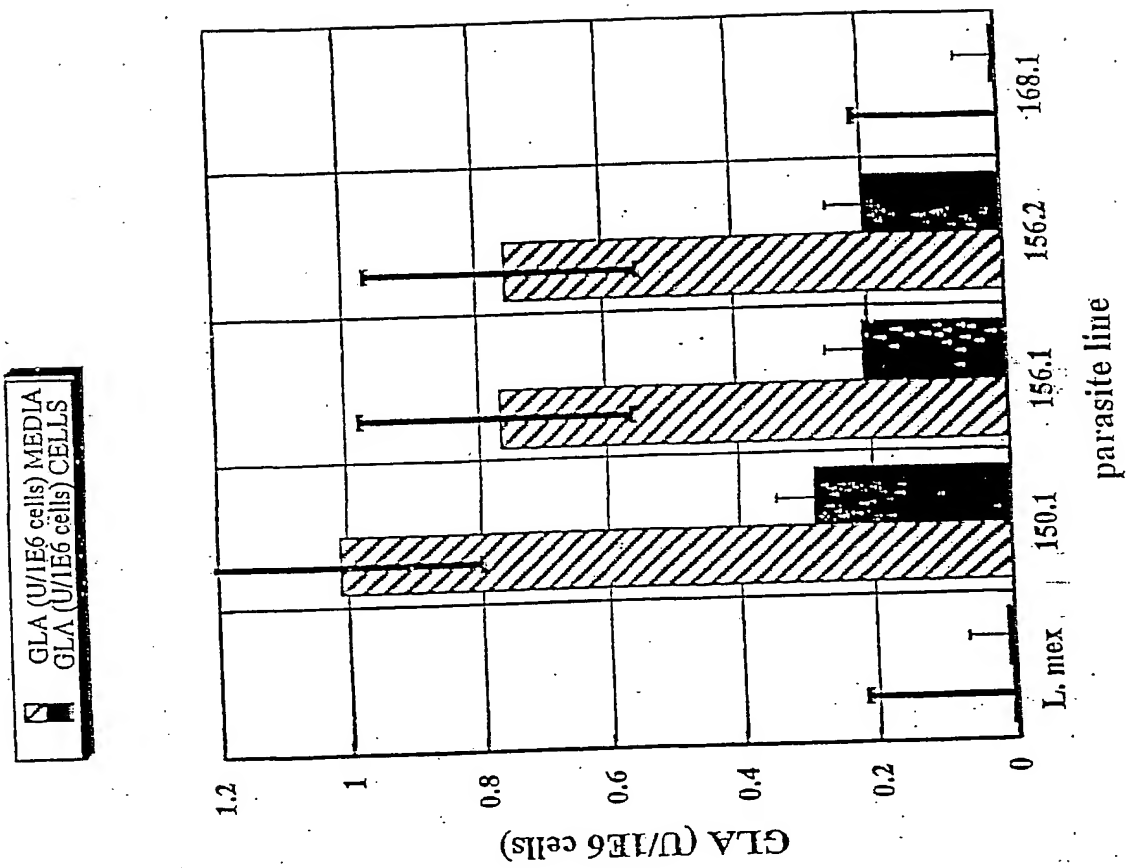
GCCGCAGTCACCGTTGCTGTCGGCACCGCGGCCGCGTGGGCAC

418

A A V T V A V G T A A A W A

Figure 1B

GLA EXPRESSION IN PROMASTIGOTES



(U = nmoles/hour)
(1E6 = 10^6)

Figure 2

GLA EXPRESSION IN PROMASTIGOTES

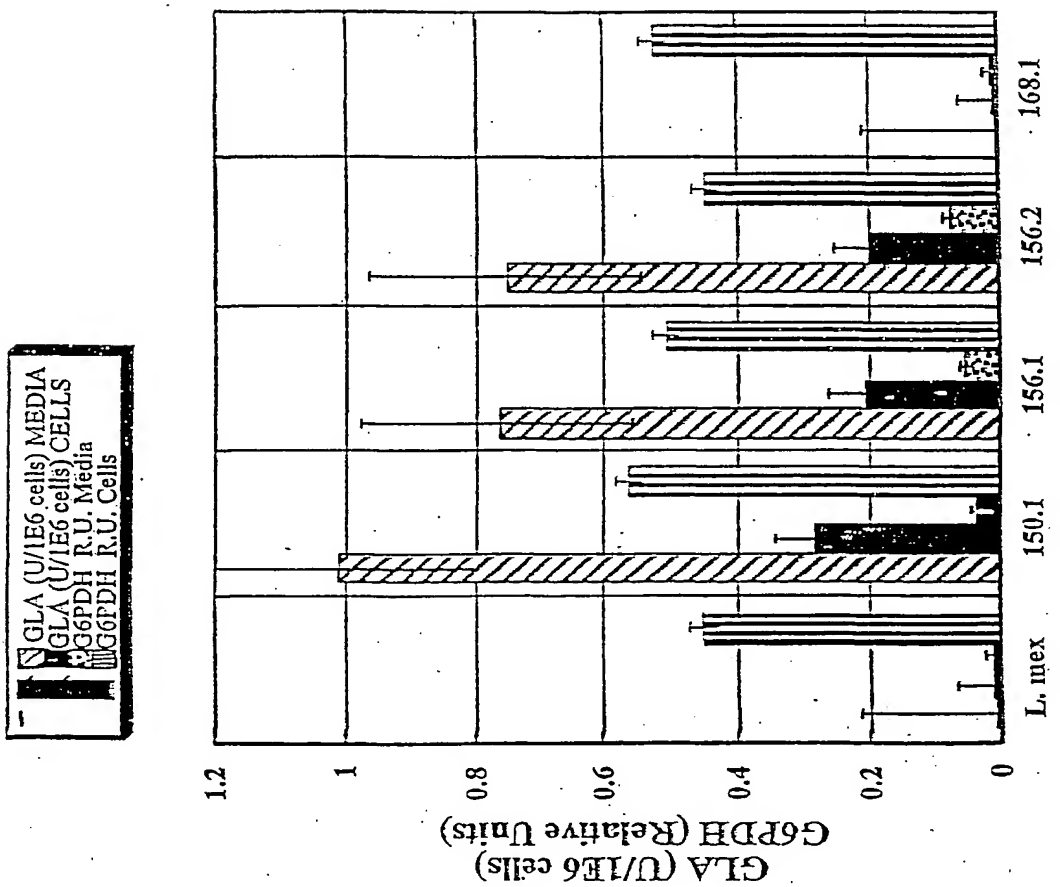


Figure 3

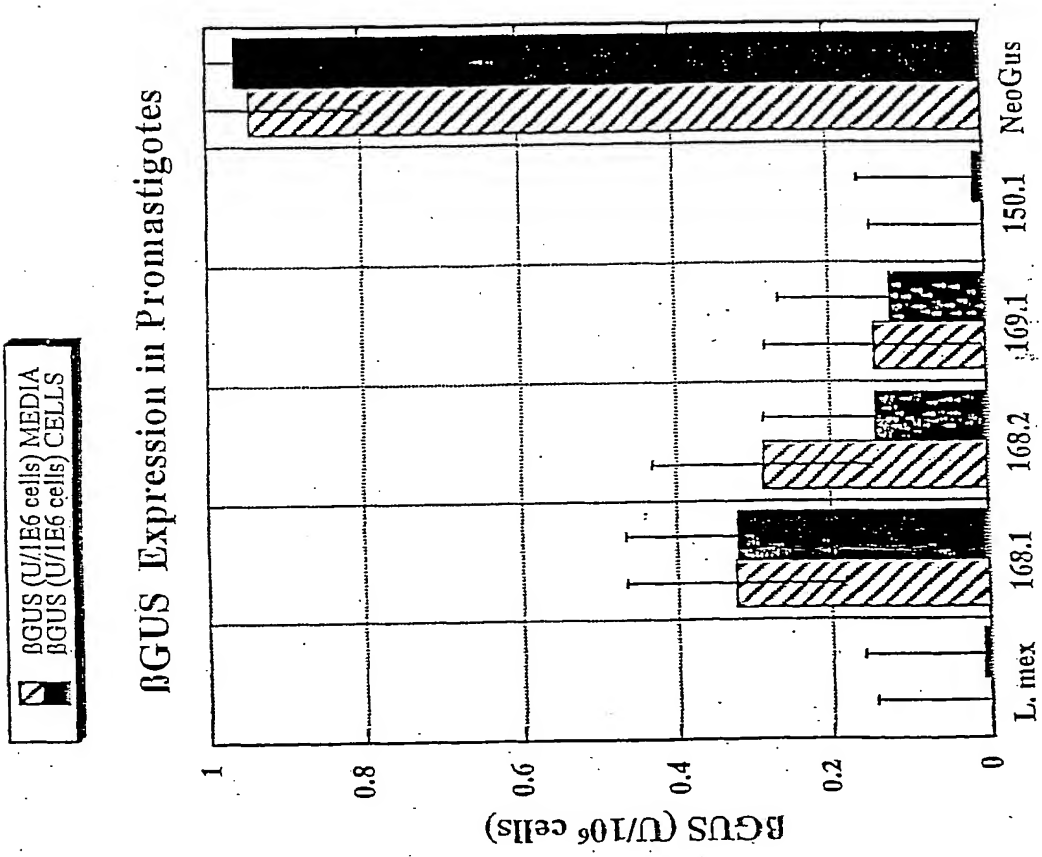


Figure 4

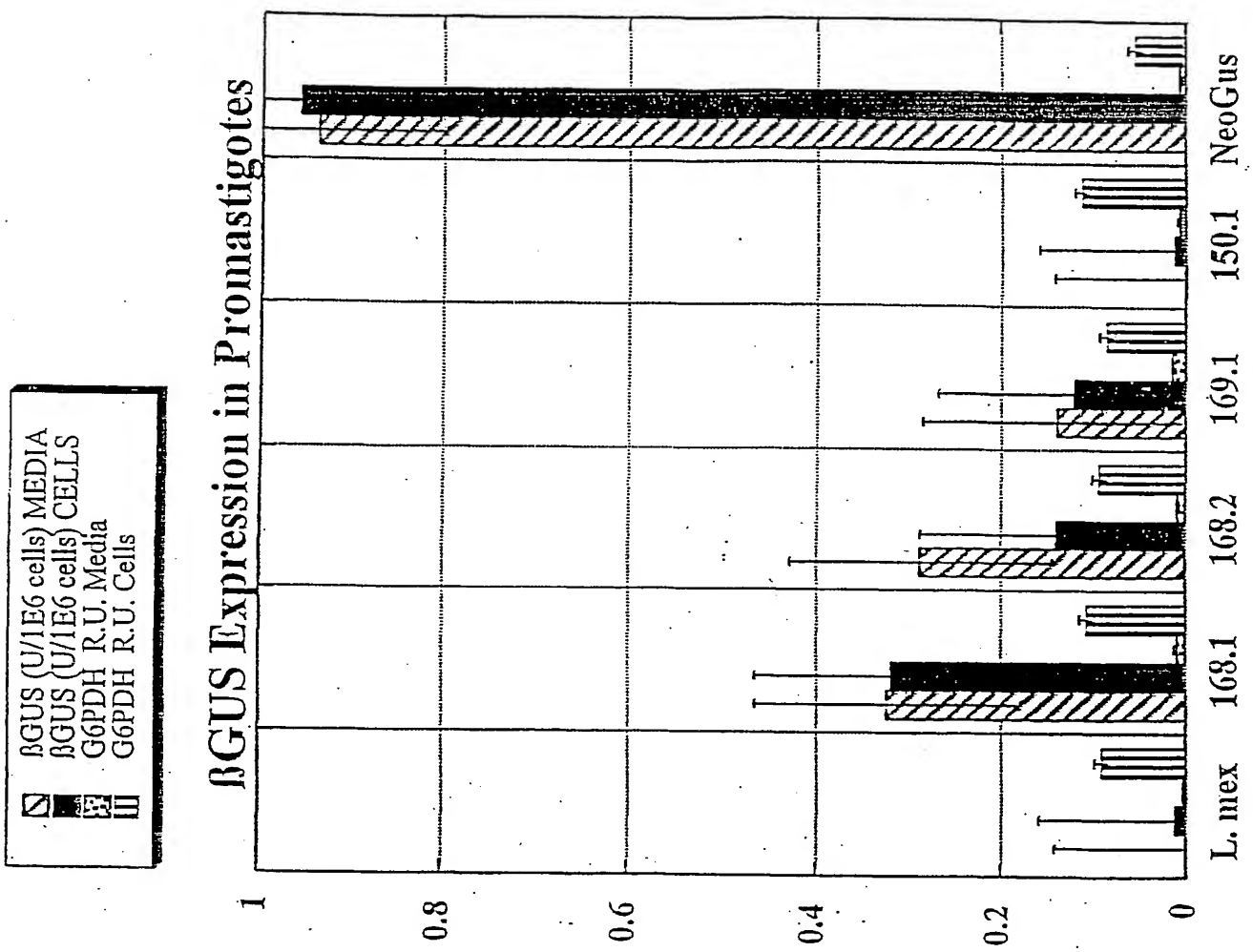


Figure 5

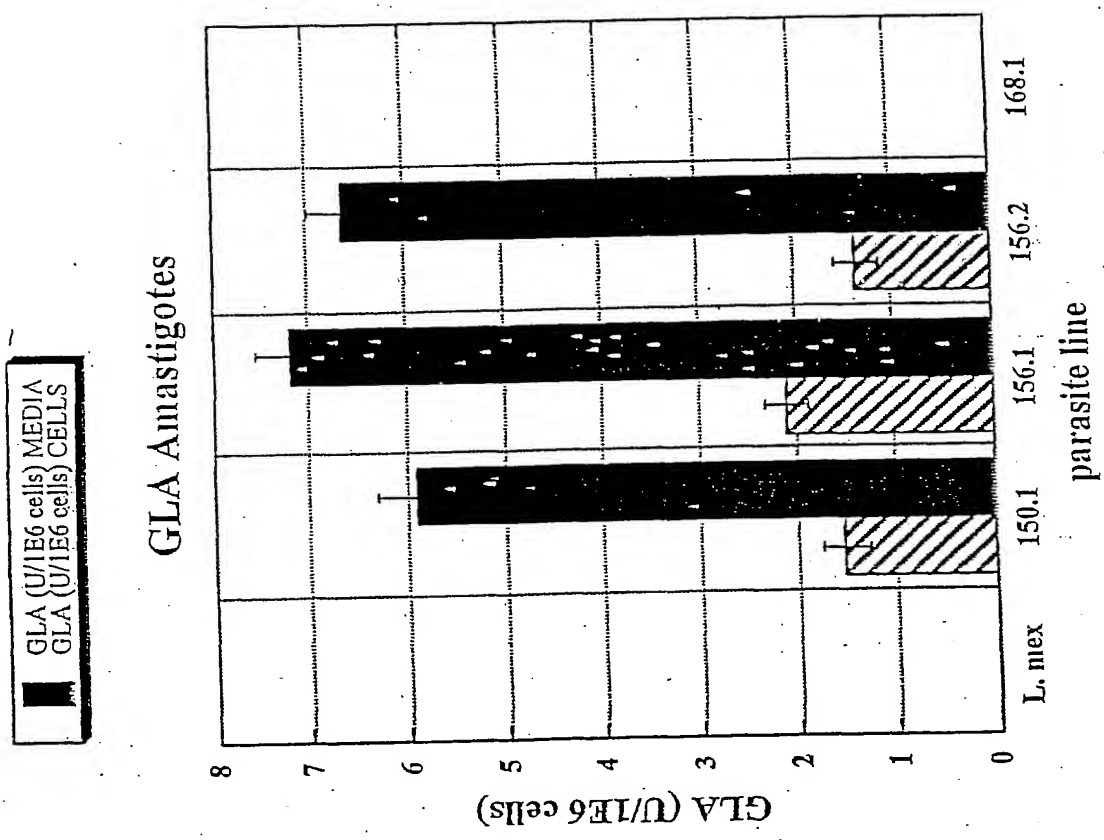


Figure 6

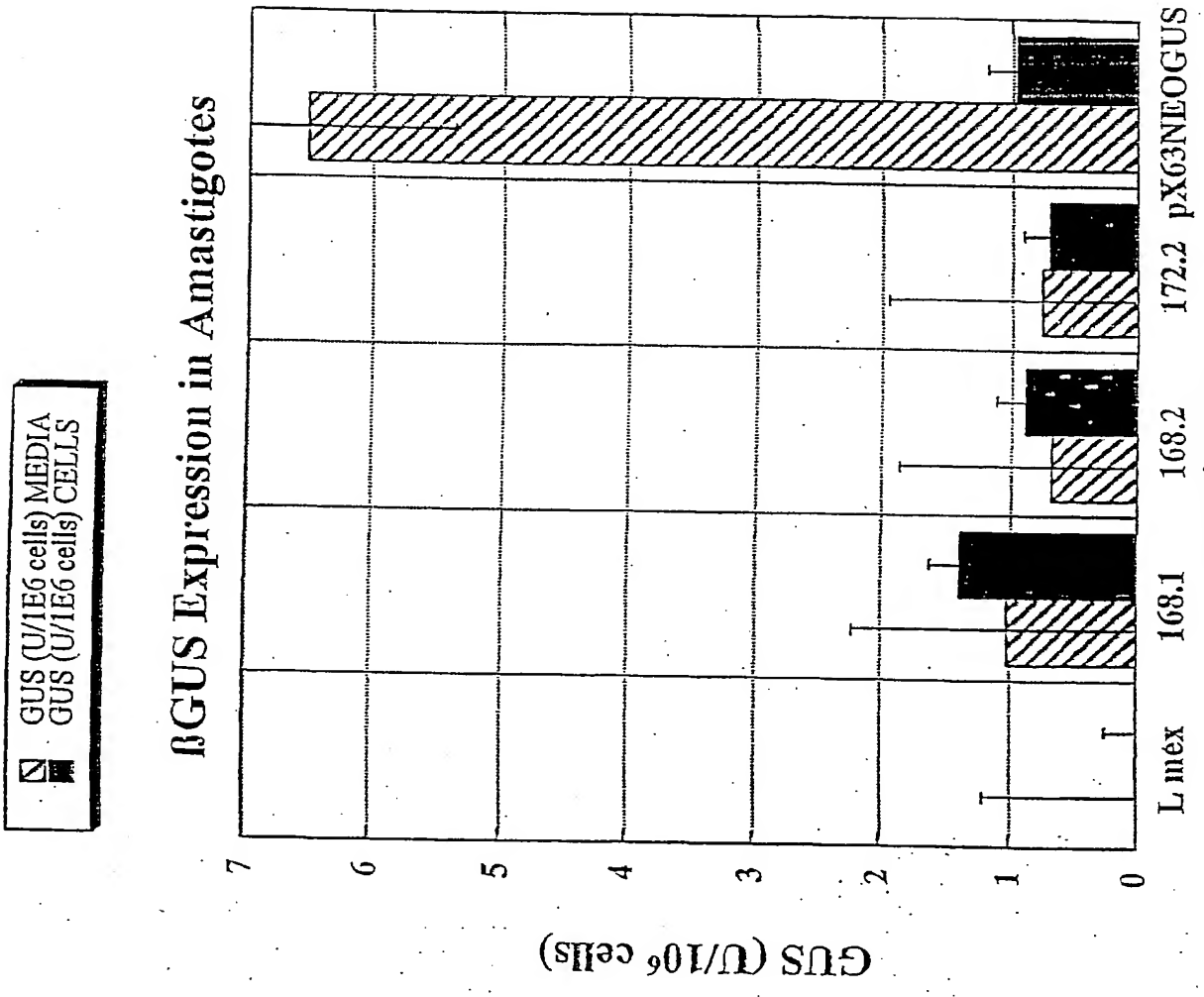


Figure 7

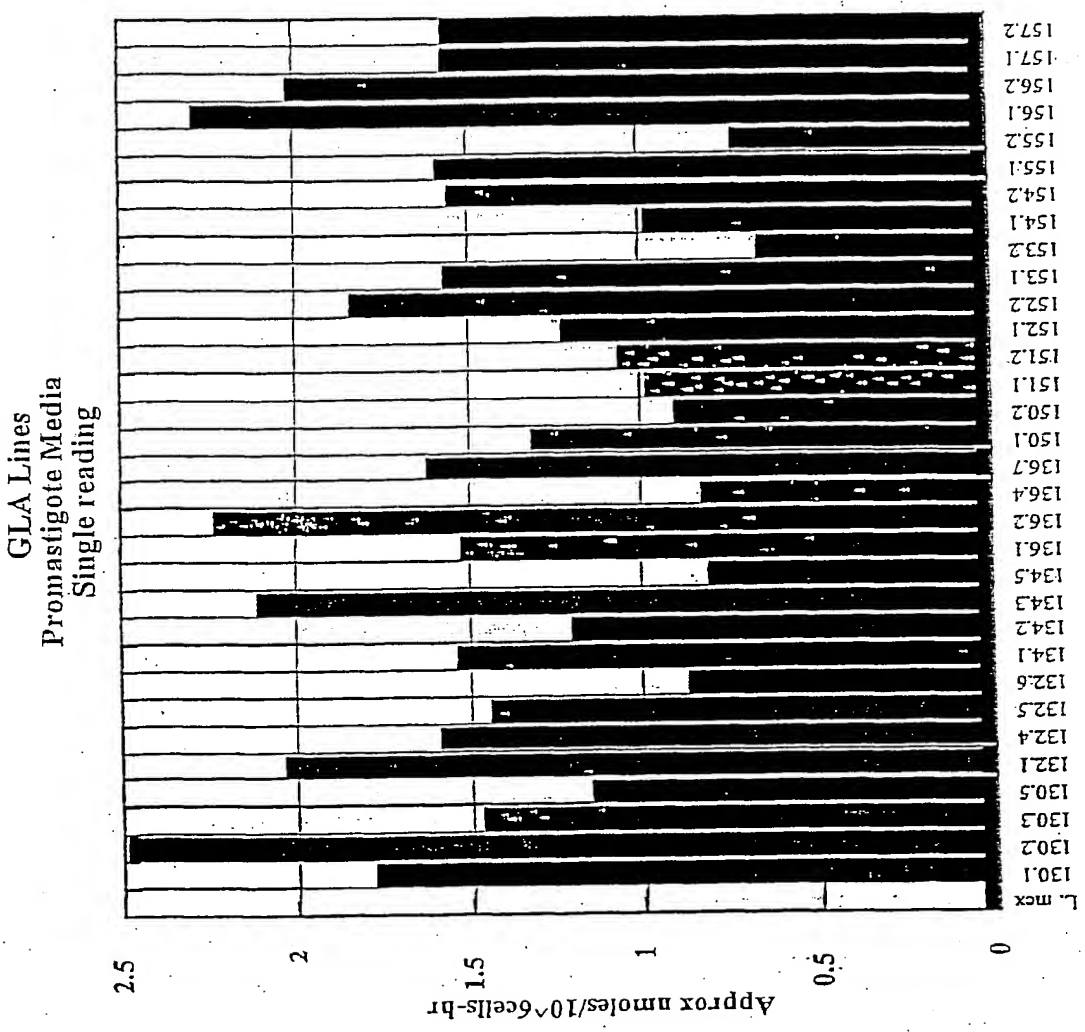


Figure 8

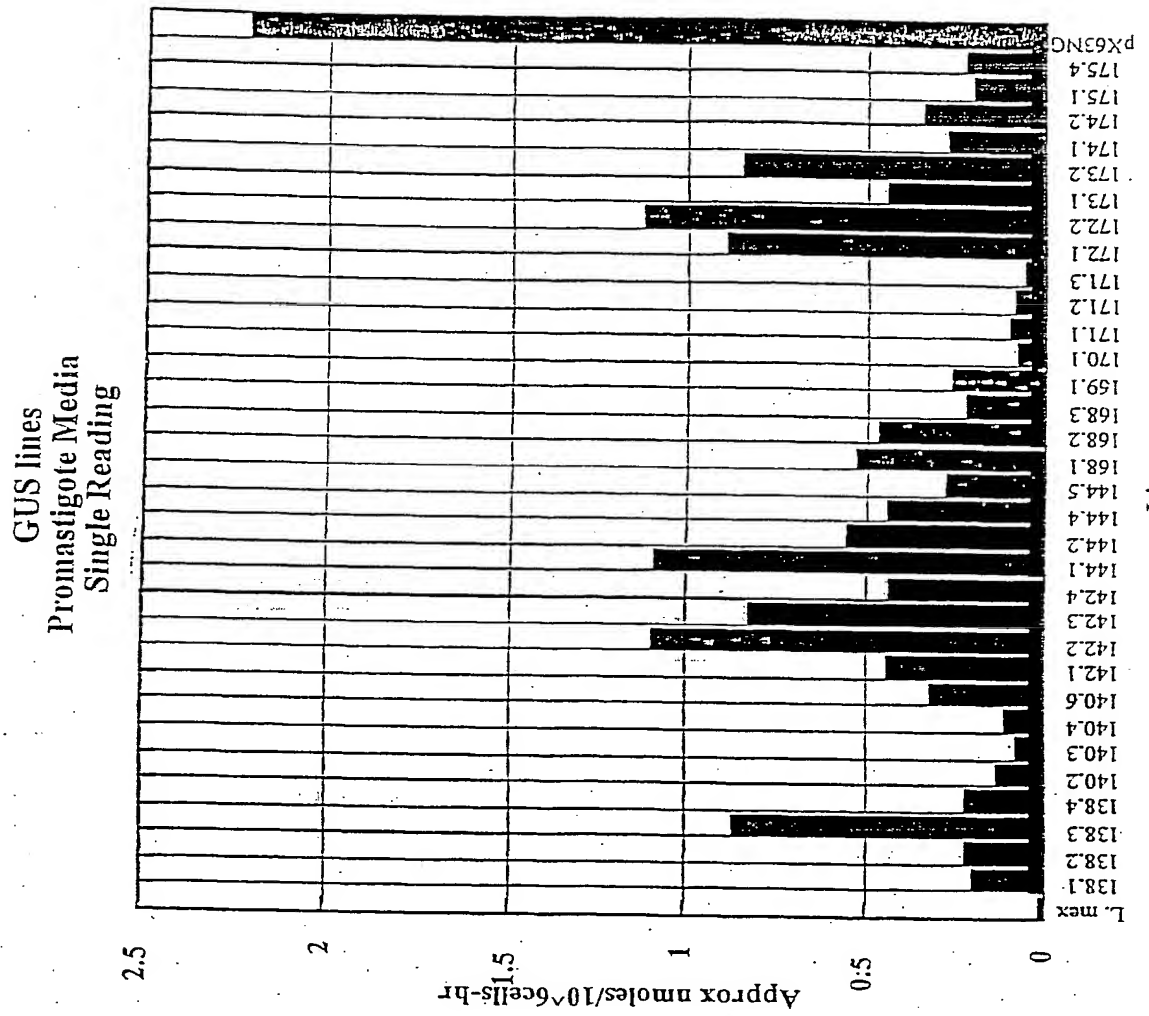


Figure 9

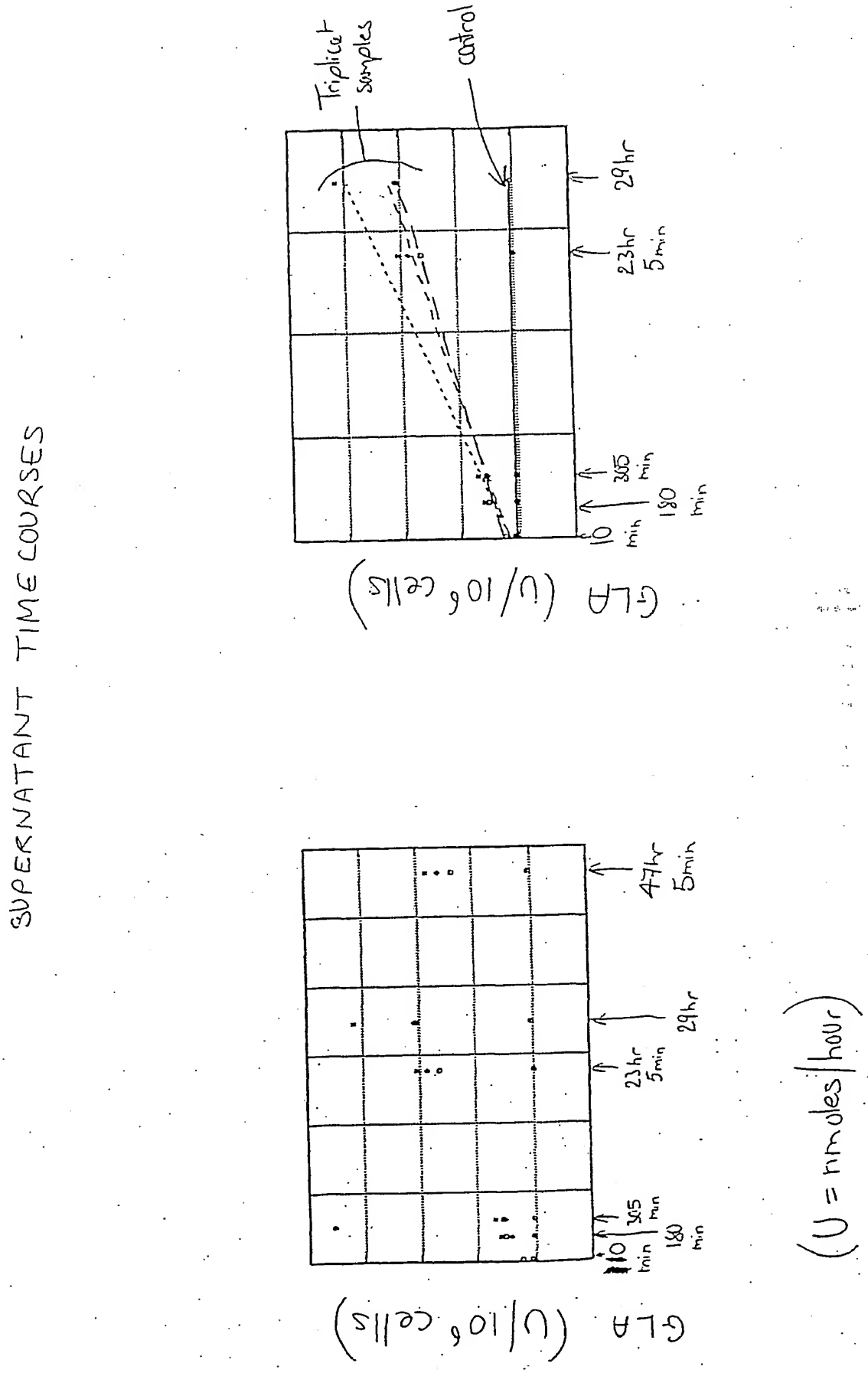


Figure 10A

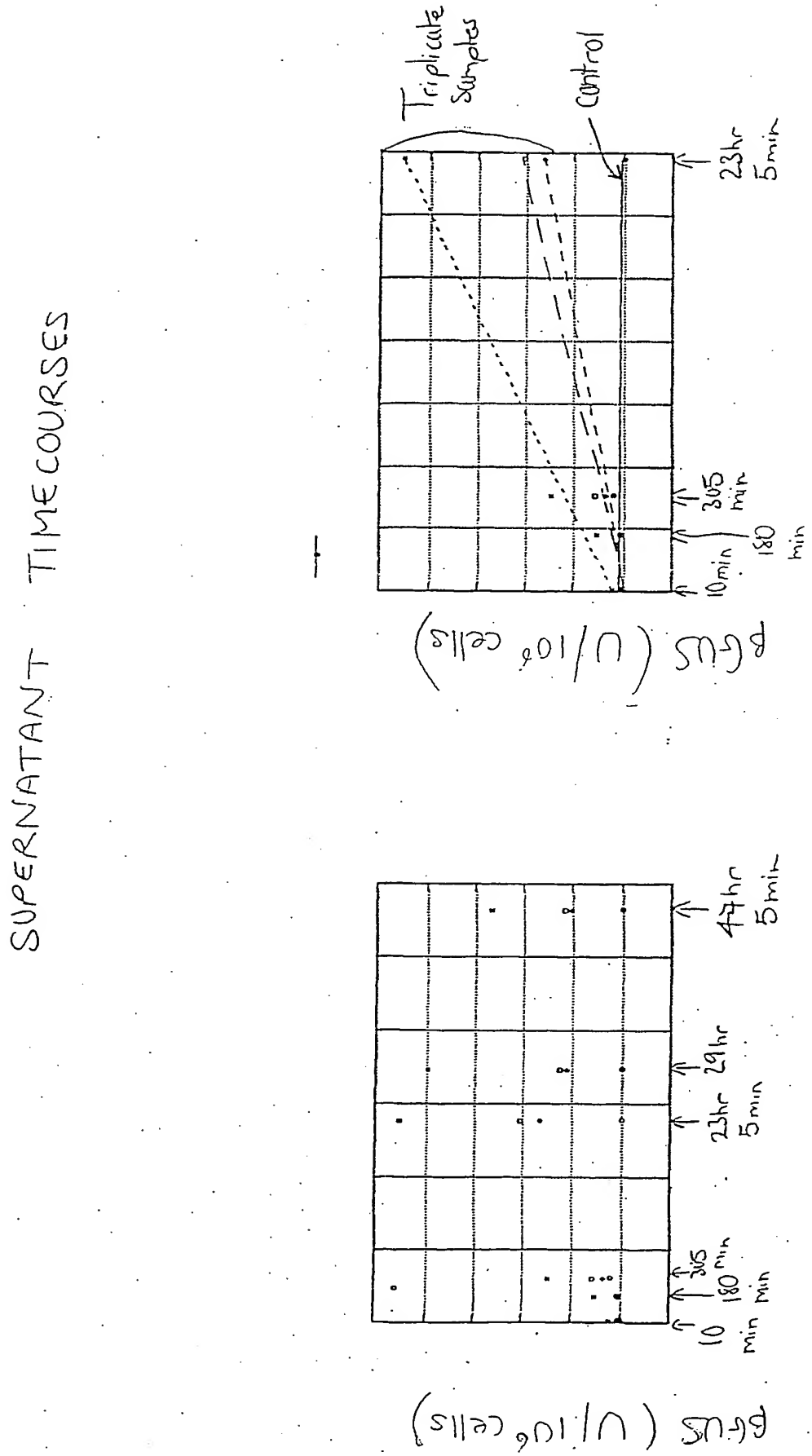


Figure 10B

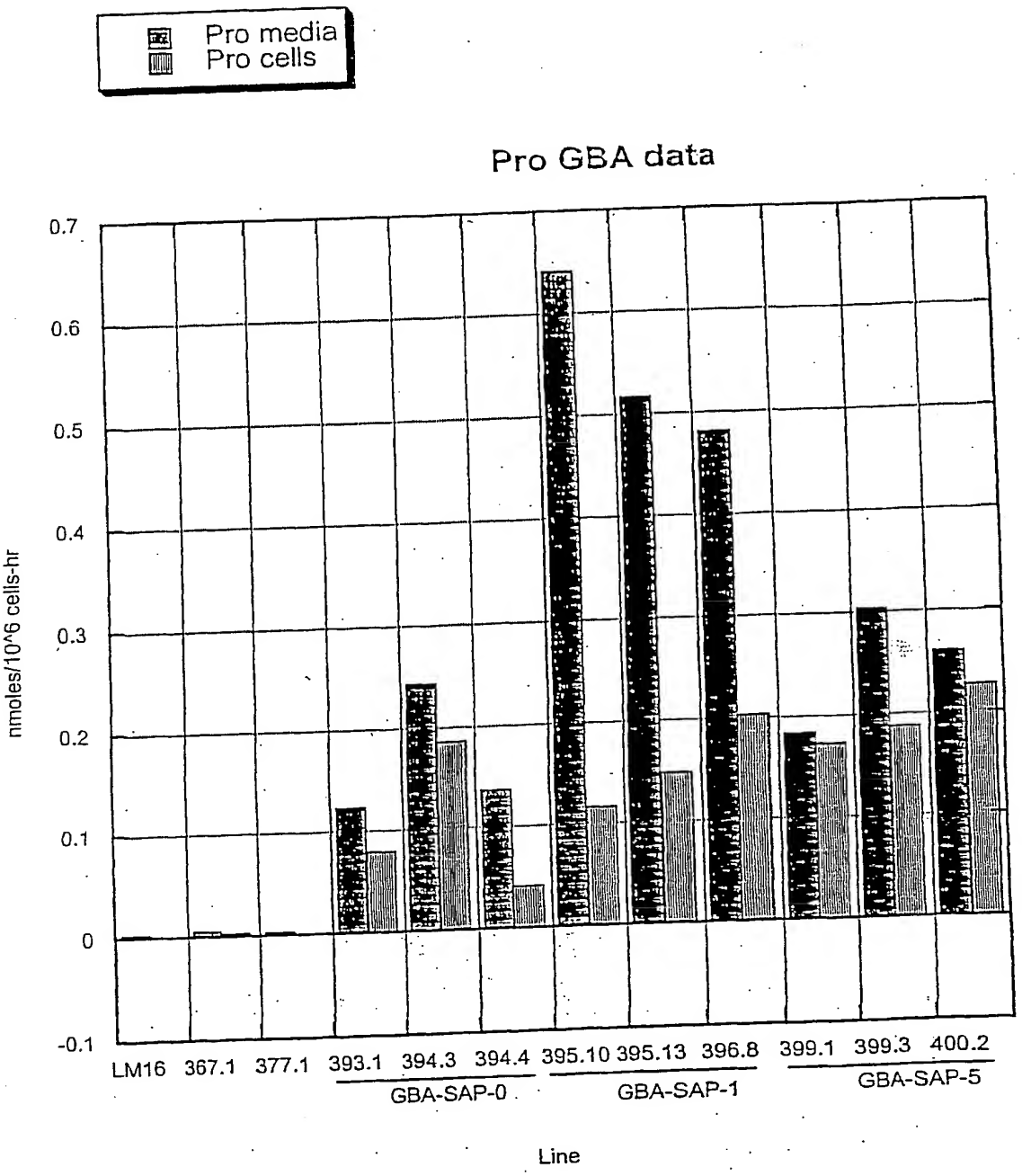


Figure 11

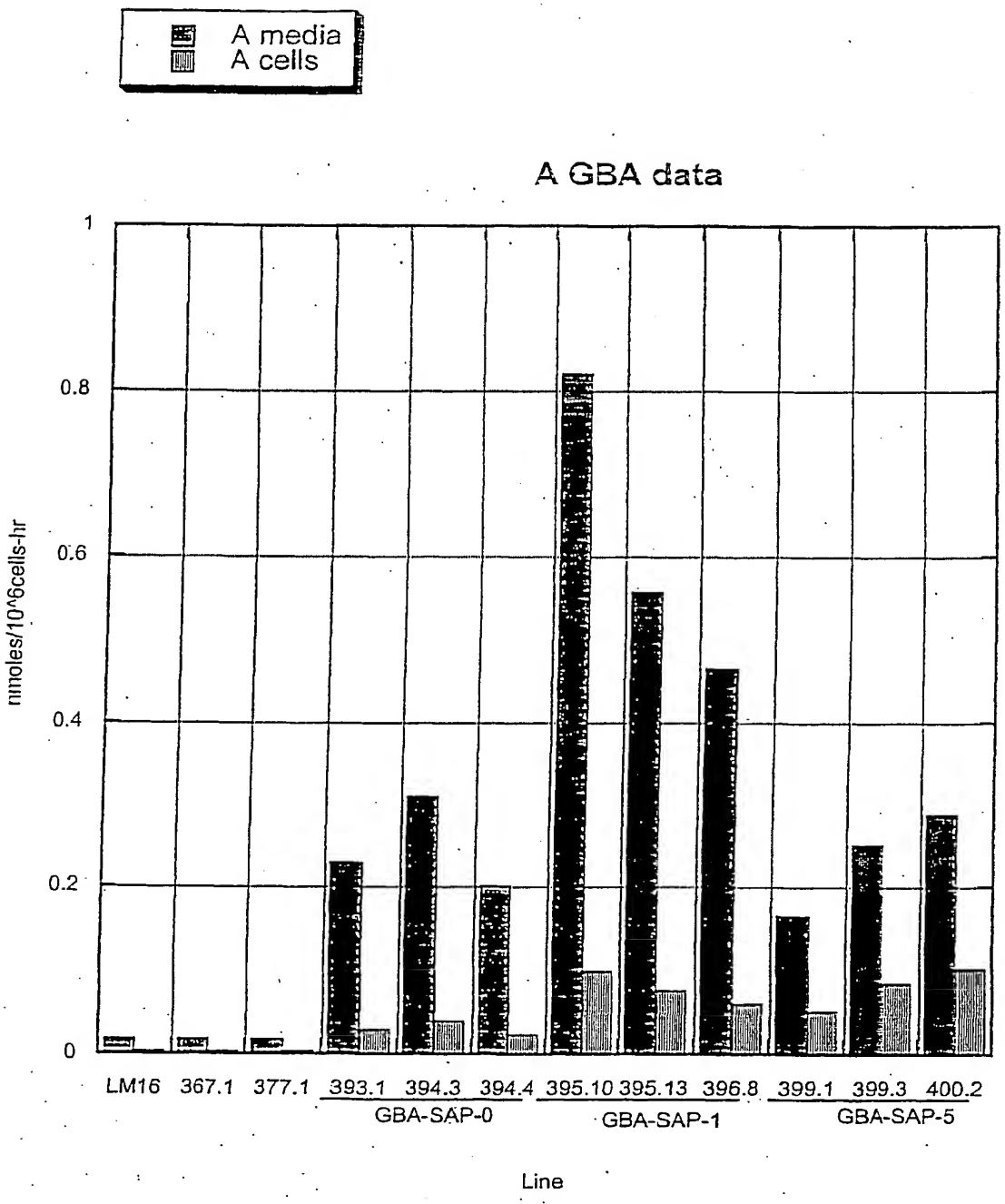


Figure 12

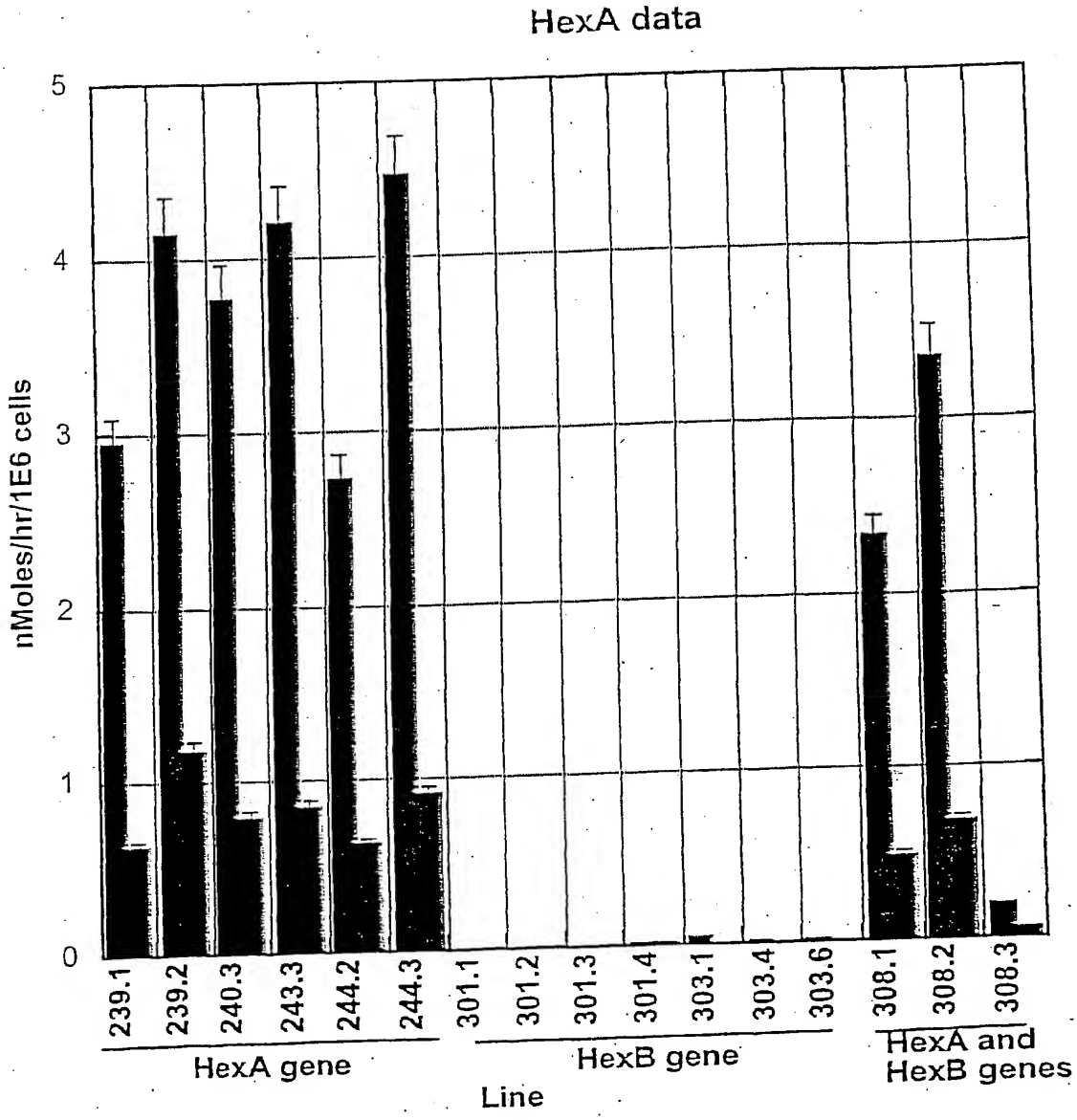


Figure 13

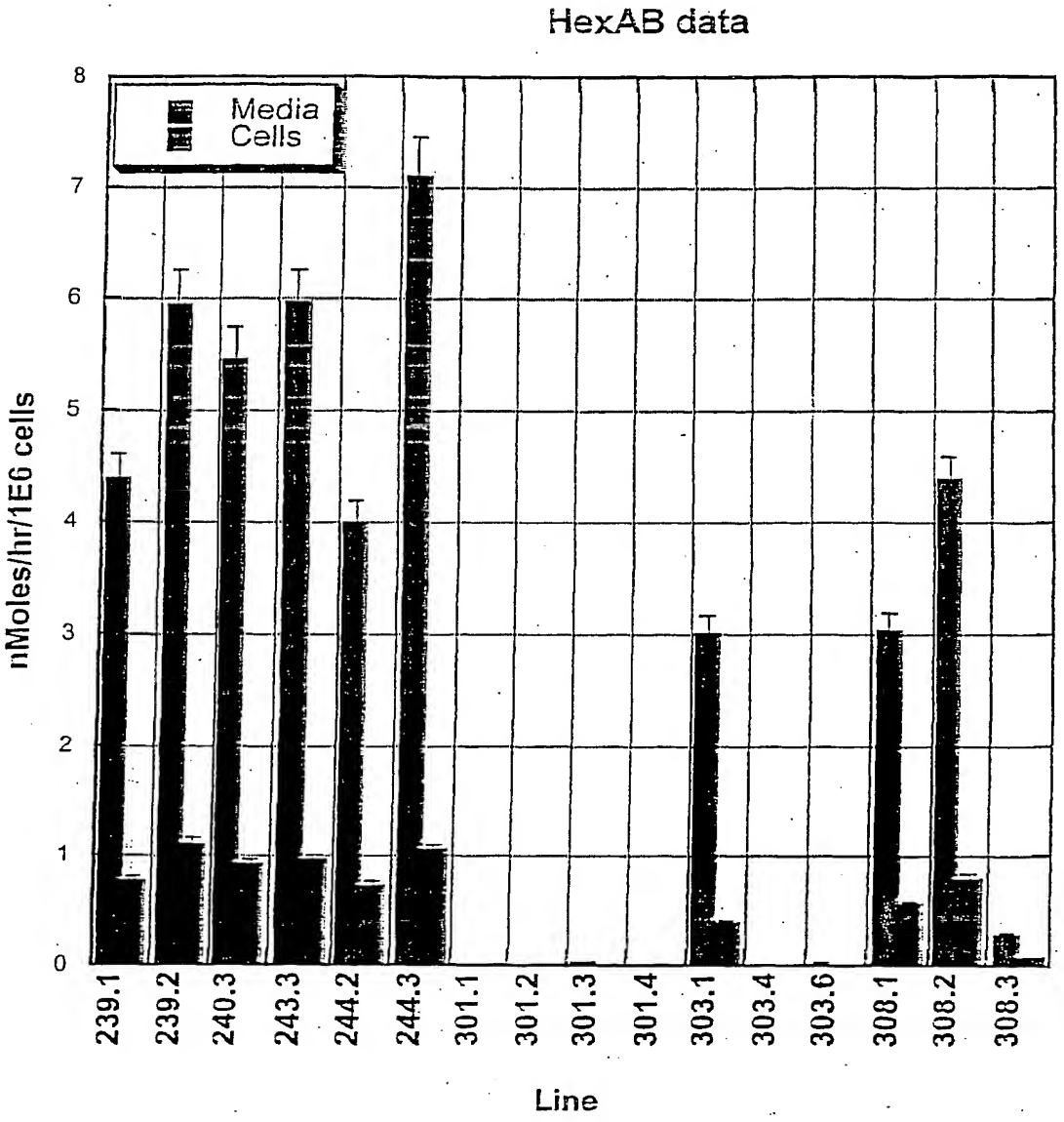


Figure 14

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(54) Title: PROTOZOAN EXPRESSION SYSTEMS FOR LYSOSOMAL STORAGE DISEASE GENES

(57) Abstract: Methods and devices are provided for expressing and secreting gene products from protozoa. The methods and devices are useful for expressing and isolating lysosomal storage disease enzymes from protozoa grown in culture, and particularly from trypanosomatids. The post-translational modification of isolated expression products can be adapted for administration to mammalian organisms. In addition, expression products can be isolated from serum free cultures thereby avoiding contamination by infectious agents such as prions. The methods and devices are also useful for delivering expression products such as lysosomal storage disease enzymes to mammalian organisms *in vivo*.

PROTOZOAN EXPRESSION SYSTEMS FOR LYSOSOMAL STORAGE DISEASE GENES

RELATED APPLICATIONS

[0001] This application claims priority to, and the benefit of U.S.S.N. 60/250,446 filed on November 30, 2000, U.S.S.N. 60/250,444 filed on November 30, 2000, and U.S.S.N. 60/290,281 filed on May 11, 2001, the disclosures of which are incorporated by reference herein in their entirety.

FIELD OF THE INVENTION

[0002] The invention relates generally to the field of pharmaceutical devices. The invention relates to methods and compositions for producing and delivering pharmaceutical products to a patient. In particular, the invention relates to methods and compositions for producing pharmaceutical products to treat lysosomal storage diseases, and to methods for administering pharmaceutical products to patients suffering from lysosomal storage diseases.

BACKGROUND

[0003] Lysosomal Storage Diseases (LSD) are caused by one or more metabolic errors that result in an abnormal accumulation or processing of material in lysosomes. Lysosomal storage diseases are usually inherited, and can be severely debilitating. At least 50 different types of lysosomal storage diseases have been identified in humans and animals. Each disease is characterized by a unique set of symptoms resulting from a loss of normal lysosomal function affecting one or more areas of the body of an individual. Symptoms may include mental and/or physical disabilities and often result in a shortened lifespan.

[0004] Most lysosomal storage diseases are caused by a deficiency in an enzyme that processes one or more products of cellular metabolism. Each disease is

characterized by a defect in a specific enzyme. Usually, the enzyme defect results from a mutation that either deletes the gene encoding the enzyme, reduces expression of the enzyme, or affects the normal function of the enzyme.

[0005] There is therefore a need in the art for devices and methods to correct the enzyme deficiencies associated with lysosomal storage diseases.

SUMMARY

[0006] The invention provides methods and devices for producing a gene product for administration to a mammal. According to the invention, a gene product is produced in a unicellular organism that is adapted for survival or growth in a host cell, tissue or organism. The gene product is preferably processed so that it is compatible with the host.

[0007] Methods and devices of the invention are useful to provide an enzyme or enzyme product to a host. Methods of the invention are particularly useful to provide an enzyme or enzyme product to the lysosomes of a patient suffering from a lysosomal storage disease.

[0008] In one aspect of the invention, a gene product is synthesized using a microorganism in an *in vitro* culture. In another aspect of the invention, a gene product is synthesized *in vivo* by a microorganism that has been administered to a host cell, tissue or organism.

[0009] A preferred microorganism of the invention is a protozoan. In a preferred embodiment, a protozoan is modified to reduce its virulence.

[0010] Preferred gene products of the invention are proteins. A preferred protein has a biological function similar to the function that is missing in a patient with a lysosomal storage disease. A most preferred protein is an enzyme that is defective in a patient suffering from a lysosomal storage disease.

[0011] According to one aspect of the invention, a gene product is expressed using a microorganism that is adapted to survive and/or grow in a host. In one embodiment, a gene product is encoded on extrachromosomal nucleic acid in the

microorganism. In another embodiment, a gene product is encoded on chromosomal nucleic acid in the microorganism.

BRIEF DESCRIPTION OF THE FIGURES

[0012] FIG. 1A shows plasmid pIR1SAT, an expression plasmid.

[0013] FIG. 1B shows plasmid pSPITS, a Signal Peptide Increased Translation and Secretion plasmid.

[0014] FIG. 2 shows expression of GLA in promastigote cultures of *L. mexicana* cell lines, the amount of GLA released into the culture medium and the amount of cellular GLA are shown for wild-type *L. mexicana* (*L. mex*), three independent *L. mexicana* cells lines transfected with a GLA expression construct (cell lines 150.1, 156.1, and 156.2), and a negative control transfected with a βGUS expression construct (cell line 168.1).

[0015] FIG. 3 shows the GLA expression results of FIG. 2, and also shows, for each cell line, the amount of G6PDH (a cytosolic marker) present in the medium relative to the amount of G6PDH associated with the cells.

[0016] FIG. 4 shows expression of βGUS in promastigote cultures of *L. mexicana* cell lines, the amount of βGUS released into the culture medium and the amount of cellular βGUS are shown for wild-type *L. mexicana* (*L. mex*), three independent *L. mexicana* cells lines transfected with a βGUS expression construct (cell lines 168.1, 168.2, and 169.1), and a negative control transfected with a GLA expression construct (cell line 150.1). The expression of βGUS from an episomal construct in *L. major* is also shown (cell line NeoGus).

[0017] FIG. 5 shows the βGUS expression results of FIG. 4, and also shows, for each cell line, the amount of G6PDH (a cytosolic marker) present in the medium relative to the amount of G6PDH associated with the cells.

[0018] FIG. 6 shows expression of GLA in amastigote cultures of *L. mexicana* cells lines, the amount of GLA released into the culture medium and the

amount of cellular GLA are shown for wild-type *L. mexicana* (*L. mex*), three independent *L. mexicana* cells lines transfected with a GLA expression construct (cell lines 150.1, 156.1, and 156.2), and a negative control transfected with a βGUS expression construct (cell line 168.1).

[0019] FIG. 7 shows expression of βGUS in amastigote cultures of *L. mexicana* cell lines, the amount of βGUS released into the culture medium and the amount of cellular βGUS are shown for wild-type *L. mexicana* (*L. mex*), three independent *L. mexicana* cells lines transfected with a βGUS expression construct (cell lines 168.1, 168.2, and 169.1), and a negative control transfected with a GLA expression construct (cell line 150.1). The expression of bacterial βGUS from an episomal construct in *L. major* is also shown (cell line NeoGus).

[0020] FIG. 8 shows GLA expression in independent isolates of *L. mexicana* cell lines transfected with a GLA expression construct.

[0021] FIG. 9 shows βGUS expression in independent isolates of *L. mexicana* cell lines transfected with a βGUS expression construct.

[0022] FIG. 10A shows a time course of GLA release into the medium for an *L. mexicana* cell line transfected with a GLA expression construct.

[0023] FIG. 10B shows a time course of βGUS release into the medium for an *L. mexicana* cell line transfected with a βGUS expression construct.

[0024] FIG. 11 shows GBA expression in promastigote *Leishmania* lines containing GBA-SAP constructs; LM16 is wild type *L. mexicana;* 367.1 and 377.1 are lines containing GBA cassettes cloned into the Xba or Bgl site of pIR1-SAT.

[0025] FIG. 12 shows GBA expression in amastigote *Leishmania* lines containing GBA-SAP constructs; LM16 is wild type *L. mexicana*; 367.1 and 377.1 are lines containing GBA cassettes cloned into the Xba or Bgl site of pIR1-SAT.

[0026] FIG. 13 shows HexA expression in independently isolated *Leishmania* strains; HexA activity is measured using substrate MUGS; promastigote lines containing the HexA, HexB, or HexA and HexB genes are indicated.

[0027] FIG. 14 shows HexAB expression in independently isolated *Leishmania* strains; HexAB activity is measured using substrate MUG.

DETAILED DESCRIPTION

[0028] The present invention provides methods and compositions for producing a gene product for a use in a pharmaceutical or therapeutic treatment. The invention is useful to produce a gene product in a form that is biologically compatible with an organism to which the gene product is intended to be administered. The invention is also useful to deliver a gene product directly to a patient. The invention is particularly useful to treat a patient with a lysosomal storage disease.

[0029] In general, methods of the invention involve expressing a gene product in a unicellular organism, preferably a protozoan. In one aspect of the invention, the gene product is obtained or purified from an *in vitro* culture of the unicellular organism. In preferred embodiments, the gene product is a therapeutic gene product that is administered to a patient. In another aspect of the invention, the unicellular organism is itself administered to a patient, and acts as a delivery device for the gene product.

[0030] Protozoans are eukaryotic organisms and the post-translational modifications of gene products expressed in protozoans are characteristic of eukaryotic post-translational modifications (e.g. patterns of protein glycosylation). Accordingly, protozoans are useful vehicles for expressing gene products to be administered to eukaryotes, including mammals.

[0031] Useful protozoans include the groups of protozoans listed in Table 1. Useful protozoans include free-living protozoans and protozoans that interact with a host organism during at least part of their life cycle. This interaction may be parasitic, symbiotic, or commensal. Parasitic organisms are preferably non-pathogenic or avirulent. Particularly useful protozoans are those that are adapted for growth and survival in mammals. These organisms are characterized by post-translational modification that is biologically compatible with their mammalian host. Accordingly, a therapeutic gene product that is expressed in one of these organisms will be suitable for administration to a mammalian host.

[0032] The predominant N-linked carbohydrate found on glycoproteins produced by Trypanosomatids are of the high mannose type. The number of mannose residues and their configuration vary amongst members of this family. This variation in mannose structure may be a consideration in choosing the appropriate strain for *in vitro* and *in vivo* production of LSD proteins. In one embodiment, preferred trypanosomatid species for protein production are those that yield proteins with carbohydrates conferring the greatest affinity for the mannose receptor thereby directing LSD proteins to the lysosomes of cells of the macrophage lineage. Candidate species can be evaluated by assaying uptake of test proteins in J774-E cells, a macrophage cell line that expresses the mannose receptor as described in Example 11.

[0033] Another consideration is the ability of the expressed enzyme to serve as a substrate for mannose-6-phosphate modification. In one embodiment of the invention, a high mannose carbohydrate can serve as a substrate for the GlcNac phosphotransferase that is the first enzyme in the two step pathway for M6P modification. According to the invention, when it is desirable to modify an expression product for generating uptake competency via interaction with an M6P receptor, it is useful to consider the distinct structures of high mannose carbohydrates associated with expression in different protozoan organisms.

[0034] According to the invention, protein modification can be done *in vitro* after purification of the protein, or *in vivo* by engineering a trypanosomatid organism to express a relevant genes of the modification pathway.

[0035] According to the invention, LSD proteins secreted from *Leishmania* contain carbohydrate with terminal mannose residues, as shown in Example 9. In one aspect of the invention, other enzymes involved in glycosylation are expressed in a suitable trypanosomatid such as *Leishmania* in order to tailor carbohydrate patterns for desirable properties. For example, expression of a *T. cruzi* trans-sialidase in *Leishmania* can be useful to produce recombinant LSD proteins with carbohydrates containing terminal sialic acid residues. This modification could also have the beneficial property of increasing the half-life of the LSD protein in the circulation of patients.

Table 1 Useful protozoans.

Group	Common Name	Typical representatives	Habitats	Common diseases
Mastigophora	Flagellates	*Trypanosoma, Giardia, Leishmania*	Freshwater; parasites of animals	African sleeping sickness, giardiasis, leishmaniasis
Sarcodina	Amoebas	*Amoeba, Entamoeba*	Freshwater and marine; animal parasites	Amoebic dysentery (Amebiasis)
Ciliophora	Ciliates	*Balantidium, Paramecium*	Freshwater and marine; animal parasites; rumen	Dysentery
Sporozoa	Sporozoans	*Plasmodium, Toxoplasma*	Primarily animal parasites; insects (vectors for parasitic diseases)	Malaria, toxoplasmosis

[0036] The invention provides methods and compositions for engineering protozoa to express gene products that are of therapeutic interest. Accordingly, methods of the invention are useful to overcome general problems associated with the development of a production system for a gene product that is to be used as a pharmaceutical or therapeutic agent. In particular, the invention provides i) a gene product in a form that is biologically compatible with the intended patient tissue or organism, ii) a purified gene product that is free of pathogens including prions, and iii) a delivery system that can target patient cells, fluids, and/or tissues. In preferred embodiments, methods and compositions of the invention are used in treatments for lysosomal storage diseases. According to the invention, a lysosomal storage disease (LSD) gene is expressed in a protozoan organism. In one embodiment, the LSD gene product is purified from the organism grown in culture, and the gene gene product is administered to a patient suffering from the corresponding LSD. According to the invention, a LSD gene product may be purified from cells, from a cell culture supernatant, or both. In another embodiment, the protozoan expressing the LSD gene is administered to the patient suffering from the LSD, and LSD gene expression in the protozoan rescues the lysosomal storage defect. Other aspects of the invention will be apparent from the present disclosure.

I. Unicellular Organisms

[0037] According to the invention, a gene product is expressed in a unicellular organism. Preferred unicellular organisms of the invention are eukaryotic organisms, and most preferably protozoans. In one embodiment, a preferred organism is non-pathogenic or avirulent. Accordingly, commensal and symbiotic organisms are useful organisms. In another embodiment, a preferred organism is an attenuated organism. An attenuated organism is preferably an organism with reduced viability in its host. Accordingly, an attenuated pathogen or parasitic organism may be useful in a method of the invention. In an alternative embodiment of the invention, a pathogenic protozoan can be used to deliver a therapeutic protein, preferably an LSD enzyme, to a patient, if the benefit of providing the therapeutic protein outweighs the side effects of the pathogen.

[0038] As used herein, the term protozoan includes any unicellular protozoan suitable for use in the devices and methods of the present invention. Haploid and diploid, including asexual diploid, protozoa are contemplated. Particularly preferred protozoa are parasitic protozoa, especially if they are non-pathogenic or avirulent, either naturally or through genetic manipulation. Set forth below is a non-limiting list of those unicellular protozoa contemplated to be within the scope of the present invention. Also set forth below is a discussion of generally preferred features and characteristics of the unicellular organisms most suitable for use in the present invention.

[0039] As discussed herein, currently preferred protozoans are Trypanosomatidae, including *Leishmania* and *Viannia* species. Currently preferred genera include *Leishmania, Trypanosoma, Toxoplasma, Plasmodium, Eimeria, Cryptosporidia, Giardia, Entamoeba,* Acanthamoeba, *Naegleria, Microspoidia,* and *Trichomona*. Most currently preferred are the species *L. major, L. tropica, L. aethiopica, L. enrietti, L. panamaeiisis, L. guyanensis, L. donovani, L. chagasi, L. infantum, L. tarentolae, L. mexicana, L. amazonesis, L. braziliensis, T. cruzi, T. brucei* and members of the trypanosomatid genus *Endotrypanum* such as *E. monterogei* and *E. schaudinni*. Other currently preferred species are *T. gondii, G. lamblia, T. vaginalis* and *T. foetus*.

[0040] A particularly preferred diploid genus is *Leishmania*. A particular advantage of expressing LSD genes in *Leishmania* is that the glycosylation of proteins in *Leishmania* is believed to occur without significant addition of terminal sialic acid residues. The resulting availability of mannose residues will permit uptake of the LSD proteins by macrophages without further post-translational modification, because the presence of mannose residues targets a protein to mammalian lysosomes and to macrophages. According to the invention, a preferred expression system for *Leishmania* is based on ribosomal gene promoters under the control of RNA polymerase 1 (PolI). A PolI expression construct of the invention may be episomal (e.g. expression of HexB was obtained from an episomal PolI expression construct) or chromosomal. In one embodiment, chromosomal expression is obtained by integrating a PolI expression construct into a ribosomal gene via homologous recombination using a linearized PolI expression vector. In an alternative embodiment, a PolI expression construct is integrated (either randomLy or via homologous recombination) at a non-ribosomal genetic locus, and the recombinant gene product (e.g. an LSD enzyme) can be expressed even after the recombinant cells reach stationary phase.

[0041] A particularly preferred haploid genus is *Toxoplasma*. *Toxoplasma* is an obligate intracellular parasite. It is well known that *Toxoplasma* is culturable. All of the known protein-coding genes are present in single-copy. Gene expression in *Toxoplasma* is apparently conventional; that is, promoters are defined and thematically similar to higher eukaryotes. (See, for example, 1995 Molecular Approaches to Parasitology, pp. 211-225, Boothroyd et al. (eds. J.C. Boothroyd & R. Komuniecki; J. Wiley & Sons, N.Y.) An especially preferred species that is well-characterized is *T. gondii*. Toxoplasma is distributed world-wide and resides in various cells, tissues and fluids of the host. During certain stages, the organism can be found in the central nervous system, skeletal and cardiac muscles, and visceral organs and tissues. Domestic cats are important reservoirs for human infection of this organism. For example, human infection can be transmitted in a variety of ways: handling infected cat feces; ingestion of meat from infected animals such as pork and lamb; transplacental transmission; transfusion with infected blood; and, via organ transplantation from infected donors. While toxoplasmic encephalitis is the most common opportunistic

parasitic infection of the central nervous system in patients with AIDS, most individuals can harbor infection asymptomatically. *Toxoplasma* infection has also been shown to affect rat behavior indicating that it acts at the level of the CNS. According to the invention, *Toxoplasma* is useful for *in vivo* delivery of one or more expression products across the blood brain barrier.

[0042] Other suitable protozoans known to have human hosts include: *Entamoeba histolytica, Entamoeba dispar, Entamoeba hartmanni, Entamoeba coli; Entamoeba polecki, Endolimax nana, Iodamoeba buetschlii, Naegleria fowleri, Acanthamoeba species, Dientamoeba fragilis, Giardia lamblia, Chilomastix mesnili, Trichomonas vaginalis, Pentatrichomonas hominis, Enteromonas hominis, Balantidium coli, Blastocystis hominis, Isospora belli, Sarcocystis species, Cryptosporidium parvum, Enterocytozoon bieneusi, Toxoplasma gondii, Plasmodium vivax, Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium species, Babesia microti, B. equi, B. bigemina, Trypanosoma b. gambiense, T.b. rhodesiense, T. cruzi, T. rangeli,* and *Pneumocystis carinii.*

[0043] In one aspect of the invention, a protozoan expressing a gene product is grown *in vitro*, and the gene product is obtained from the *in vitro* culture. In a preferred embodiment, an expressed gene product is released or secreted into the culture medium. The gene product is then obtained or purified from the culture medium. In an alternative embodiment, an expressed gene product is retained within the microorganism, and the gene product is obtained or purified from the microorganism.

[0044] In another aspect of the invention, a protozoan expressing a gene product is administered to a host organism, and the gene product is expressed by the protozoan *in situ* in the host. In a preferred embodiment, the gene product is secreted from the protozoan. In an alternative embodiment, the gene product is bound to the external membrane of the protozoan. In yet a further embodiment, the gene product remains inside the protozoan, and metabolizes one or more substrates that diffuse into or are transported into the protozoan from the host. Reaction product then diffuses out, or is transported out from the protozoan to the host.

A. Organisms useful for *in vitro* production

[0045] Preferred organisms for *in vitro* growth and gene expression are microorganisms that grow in culture. A preferred microorganism is a protozoan that grows to high density *in vitro*. This is useful to obtain large amounts of the expressed gene product from the *in vitro* culture.

[0046] Preferred protozoa for *in vitro* production and secretion generally include trypanosomatids, and preferably include *Leishmania* species such as *L. tarentolae,* and *L. mexicana*, and other protozoa such as *Crithidia* which are non-pathogenic and can be grown *in vitro*. Different protozoa can be used for the in *vitro* production of expression products such as LSD enzymes in order to obtain proteins with different patterns of post-translational modification. According to the invention, different post-translational modifications may be adapted for different uses, including administering an isolated or purified expression product to mammalian organisms, as discussed below. Examples of groups of protozoa with different patterns of post-translational modification include *Trypanosoma*, *Leptomonas*, and *Herpetomonas*; *Crithidia* and *Leishmania enrietti*; *Leishmania braziliensis*, *L. amazonensis*, and *Blastocrithidia*; *Leishmania major* and *L. chagasi*; and *Leishmania tarentolae.* The pattern of post-translational modification in *Trypanosoma*, *Leptomonas*, and *Herpetomona* is similar to the pattern of post-translational modification in mammals, and recombinant proteins (e.g. LSD enzymes) isolated from *Trypanosoma*, *Leptomonas*, and *Herpetomona* are preferred substrates for phosphorylation. However, proteins isolated from other protozoa may also be phosphorylated. According to the invention, recombinant proteins can be isolated or purified from recombinant trypanosomatids (from cells or from a cell culture supernatant if the recombinant protein product is secreted) via affinity purification of a protein with a high mannose carbohydrate using for example lectin affinity chromatography (e.g. based on the interaction of mannose with ConA).

[0047] In a preferred embodiment of the invention, a microorganism is grown in culture in the absence of growth supplements derived from a mammalian source. Accordingly, the *in vitro* culture, and any gene product purified therefrom, will

be free of any mammalian contaminant. In particular the culture and purified gene product will be prion free.

[0048] A preferred prion free culture medium is a synthetic medium that does not contain serum, lymphokines or any other growth factors derived from serum. A most preferred medium is serum free M199. A most preferred organism that grows in serum free media is *Leishmania tarentolae*. *Leishmania tarentolae* was isolated from a reptile, and grows in brain heart infusion in the absence of serum or other growth supplement derived from mammals, such as fetal bovine serum or bovine embryonic fluid. However, *Leishmania tarentolae* is preferably grown in serum free synthetic medium, to avoid contamination of the culture with disease agents such as prions, which may be present in brain heart infusion and in serum. It is expected that other protozoan organisms that are free-living, or include a non-mammalian host in their life-cycle, will be readily grown in serum free synthetic culture medium. An advantage of using a protozoan expression system is that protozoan organisms do not produce prions.

[0049] Leishmania *tarentolae* is a most preferred species for the expression system, because, in addition to growing in serum free medium, it also grows to high density in an *in vitro* culture. This is particularly advantageous both from a cost standpoint as well as from a safety standpoint, because proteins produced in this culture system will be produced in the absence of any mammalian materials (thereby preventing contamination of the protein preparation by prions or other infective agents such as retroviruses). LSD gene expression constructs could be readily beintroduced into *L. tarentolae* to obtain active enzyme.

[0050] In an alternative embodiment, a prion free culture medium may include a lymphokine or other growth factor, provided that the lymphokine or growth factor is produced recombinantly and is not obtained from a mammalian serum. Accordingly, a protozoan that requires one or more lymphokines or other growth factors may be grown in a prion free culture medium. A gene product obtained from this culture will be prion free, and also free of any other potentially harmful mammalian contaminant (such as retroviruses etc.)

[0051] In other embodiments of the invention, a culture of recombinant protozoa grown in medium containing serum is diluted into serum free medium and grown for several generations. An expression product, preferably a LSD gene product, can be purified from the serum free culture. Such serum free growth and purification methods are illustrated in Example 5 and can be applied to protozoa other than *Leishmania tarentolae*. These serum free growth methods are particularly useful for preparing prion free expression products from other *Leishmania* species and other preferred organisms of the invention.

[0052] In one embodiment of the invention, a protozoan for *in vitro* gene product expression is selected such that it will not infect the mammal to which the purified gene product will be administered. In a preferred embodiment, the protozoan is an attenuated organism that is not pathogenic for humans. In a most preferred embodiment, the protozoan is not a human pathogen or parasite.

B. Organisms useful for *in vivo* production

i. General features

[0053] Preferred organisms for *in vivo* gene product expression are protozoa that survive or grow in the host to which the gene product is to be delivered. In a preferred embodiment, the protozoan is avirulent, and more preferably non-pathogenic. Useful protozoa are symbiotic or commensal organisms. Alternatively, an attenuated parasitic organism is useful to express a gene product in a host.

[0054] In one aspect of the invention, a protozoan persists in the host in order to achieve long term delivery of the expression product to the host, preferably between 1 year and several decades.

[0055] In another aspect of the invention, a protozoan only survives in the host for a short period of time, preferably between approximately 1 hour and approximately 7 days, in order to have short term delivery of the expression product and in order to minimize any long term effects caused by the presence of the protozoan in the host. Alternatively, a protozoan of the invention survives for an intermediate period of

time in the host, preferably between approximately 1 month and approximately 12 months.

[0056] Preferred attenuated organisms are conditionally defective organisms, such as auxotrophic organisms. A useful auxotrophic organism is a DHFR-TS⁻ *Leishmania* species. For example, a DHFR-TS⁻ *L. major* strain has a reduced half-life in mice, and reduced growth in macrophages.

[0057] Other examples of attenuated organisms are also useful. In general, an attenuated protozoan is generated by functionally disabling a gene required for growth and replication in the host. Examples of such genes are known in the art, for example the lpgl⁻ gene of Leishmania *major*. Knockouts of other genes such as the gp63 surface protease gene also produce attenuated organisms.

[0058] In an alternative embodiment of the invention, a protozoan is engineered to contain a suicide gene such as the HSV-thymidine kinase gene. In the presence of the appropriate drug (for example gancyclovir), the protozoan is killed. Accordingly, the persistence of a protozoan with a suicide gene can be controlled directly.

ii. Organisms for treating lysosomal storage diseases

[0059] Lysosomal storage diseases typically cause tissue specific symptoms, even though the defect is usually present in all cells of the body. For example, Gaucher disease is manifested primarily in the liver, where defective lysosomal processing in macrophages is deleterious. Many LSDs cause neurological defects due to a defective processing of lipids that are characteristic of the CNS (e.g. sphingolipids and gangliosides).

[0060] Accordingly, in one aspect of the invention, a useful protozoan is a tissue specific protozoan that will specifically target the tissue where the LSD gene defect is manifested. For example, *Toxoplasma* species are useful to target neuronal tissue, and *Leishmania* species are useful to target macrophages in the liver. In one embodiment, *Leishmania donovani* is expected to be a particularly useful protozoan for delivering an LSD gene product to macrophages in the liver.

[0061] Other useful protozoa are characterized by the following tissue specificities, and are useful to deliver LSD gene products to these tissues: skin: *Leishmania*; eye: *Acanthamoeba*; mouth: Amoebae and flagellates (usually non-pathogenic); gut: *Giardia, Entamoeba* (and invasion to liver), *Cryptosporidium, Isospora, Balantidium*; G.I. tract: *Trichomonas*; bloodstream: *Plasmodium, Trypanosoma*; spleen: *Leishmania*; liver: *Leishmania, Entamoeba*; muscle: *Trypanosoma cruzi*; CNS: *Trypanosoma, Naegleria, Toxoplasma, Plasmodium.*

[0062] In an alternative embodiment of the invention, a LSD gene is not targeted to a particular tissue, but is expressed in a protozoan that is present throughout the body. For example, many *Leishmania* species are useful to deliver a gene product to macrophages throughout the body, and more particularly to the lysosomes of the macrophages.

II. Gene Products

[0063] While methods and compositions of the invention are useful for producing and delivering any therapeutic gene product, the invention is particularly useful for gene products that overcome enzymatic defects associated with lysosomal storage diseases.

[0064] Preferred LSD genes are shown in Table 2. In a preferred embodiment, a wild-type LSD gene product is delivered to a patient suffering from a defect in the same LSD gene. In particular, a human LSD gene or enzyme is preferably delivered to a human patient. In alternative embodiments, a functional sequence or species variant of the LSD gene or protein is used. Preferred species variants are mammalian and include mouse LSD genes and proteins. In further embodiments, a gene coding for a different enzyme that can rescue an LSD gene defect is used according to methods of the invention.

Table 2. Lysosomal Storage Diseases and associated enzyme defects

A. Glycogenosis Disorders		
Disease Name	Enzyme Defect	Substance Stored
Pompe Disease	Acid-a1, 4-Glucosidase	Glycogen α 1-4 linked Oligosaccharides
B. Glycolipidosis Disorders		
Disease Name	Enzyme Defect	Substance Stored
GM1 Gangliodsidosis	β-Galactosidase	GM_1 Ganliosides
Tay-Sachs Disease	β-Hexosaminidase A	GM_2 Ganglioside
GM2 Gangliosidosis: AB Variant	GM_2 Activator Protein	GM_2 Ganglioside
Sandhoff Disease	β-Hexosaminidase A&B	GM_2 Ganglioside
Fabry Disease	α-Galactosidase A	Globosides
Gaucher Disease	Glucocerebrosidase	Glucosylceramide
Metachromatic Leukodystrophy	Arylsulfatase A	Sulphatides
Krabbe Disease	Galactosylceramidase	Galactocerebroside
Niemann-Pick, Types A and B	Acid Sphingomyelinase	Sphingomyelin
Niemann-Pick. Type C	Cholesterol Esterification Defect	Sphingomyelin
Nieman-Pick, Type D	Unknown	Sphingomyelin
Farber Disease	Acid Ceramidase	Ceramide
Wolman Disease	Acid Lipase	Cholesteryl Esters
C. Mucopolysaccharide Disorders		
Disease Name	Enzyme Defect	Substance Stored
Hurler Syndrome (MPS IH)	α-L-Iduronidase	Heparan & Dermatan Sulfates
Scheie Syndrome (MPS IS)	α-L-Iduronidase	Heparan & Dermatan, Sulfates
Hurler-Scheie (MPS IH/S)	α-L-Iduronidase	Heparan & Dermatan Sulfates
Hunter Syndrome (MPS II)	Iduronate Sulfatase	Heparan & Dermatan Sulfates

Sanfilippo A (MPS IIIA)	Heparan N-Sulfatase	Heparan Sulfate
Sanfilippo B (MPS IIIB)	α-N-Acetylglucosaminidase	Heparan Sulfate
Sanfilippo C (MPS IIIC)	Acetyl-CoA-Glucosaminide Acetyltransferase	Heparan Sulfate
Sanfilippo D (MPS IIID)	N-Acetylglucosamine –6-Sulfatase	Heparan Sulfate
Morquio A (MPS IVA)	Galactosamine-6-Sulfatase	Keratan Sulfate
Morquio B (MPS IVB)	β-Galactosidase	Keratan Sulfate
Maroteaux-Lamy (MPS VI)	Arylsulfatase B	Dermatan Sulfate
Sly Syndrome (MPS VII)	β-Glucuronidase	
D. Oligosaccharide/Glycoprotein Disorders		
Disease Name	Enzyme Defect	Substance Stored
α–Mannosidosis	α–Mannosidase	Mannose/Oligosaccharides
β-Mannosidosis	β-Mannosidase	Mannose/Oligosaccharides
Fucosidosis	α-L-Fucosidase	Fucosyl Oligosaccharides
Asparylglucosaminuria	N-Aspartyl- β-Glucosaminidase	Asparylglucosamine Asparagines
Sialidosis (Mucolipidosis I)	α-Neuraminidase	Sialyloligosaccharides
Galactosialidosis (Goldberg Syndrome)	Lysosomal Protective Protein Deficiency	Sialyloligosaccharides
Schindler Disease	α-N-Acetyl-Galactosaminidase	
E. Lysosomal Enzyme Transport Disorders		
Disease Name	Enzyme Defect	Substance Stored
Mucolipidosis II (I-Cell Disease)	N-Acetylglucosamine-1- Phosphotransferase	Heparan Sulfate
Mucolipidosis III (Pseudo-Hurler	Same as ML II	

Polydystrophy)		

F. Lysosomal Membrane Transport Disorders		
Disease Name	Enzyme Defect	Substance Stored
Cystinosis	Cystine Transport Protein	Free Cystine
Salla Disease	Sialic Acid Transport Protein	Free Sialic Acid and Glucuronic Acid
Infantile Sialic Acid Storage Disease	Sialic Acid Transport Protein	Free Sialic Acid and Glucuronic Acid
G. Other		
Disease Name	Enzyme Defect	Substance Stored
Batten Disease (Juvenile Neuronal Ceroid Lipofuscinosis)	Unknown	Lipofuscins
Infantile Neuronal Ceroid Lipofuscinosis	Palmitoyl-Protein Thioesterase	Lipofuscins
Mucolipidosis IV	Unknown	Gangliosides & Hyaluronic Acid
Prosaposin	Saposins A, B, C or D	

[0065] In one embodiment of the invention, two or more LSD genes are expressed in a protozoan cell line. In a preferred embodiment, HexA and HexB are coexpressed.

III. Choice of expression system

A. Extrachromosomal or chromosomal expression systems

[0066] According to the invention, a gene product can be expressed from either an extachromosomal (episomal) expression vector, from an expression construct that is integrated into a chromosomal locus, or a combination of both.

[0067] In a preferred embodiment, a stable genomic expression construct is used for *in vivo* delivery. In another embodiment, an episomal expression system is used for in vitro expression. In a further embodiment, a protozoan is engineered to express proteins having a desired carbohydrate structure. A protozoan is engineered to

express one or more glycosylation enzymes in addition to a therapeutic protein of interest. Accordingly, the therapeutic protein is expressed and glycosylated by the recombinant glycosylation enzymes. In a most preferred embodiment, a glycosylation enzyme is used to add mannose-6-phosphate to an expressed protein, thereby providing the expressed protein with a lysosome targeting signal in mammals.

[0068] A non-limiting example of an enzyme expressed in a protozoan is trans-sialidase, which has been expressed in *Leishmania*.

B. Transcriptional and translational control

[0069] In general, high levels of transcription and translation are useful in methods of the invention. According to preferred embodiments of the invention, the coding sequence for an expression product, preferably a lysosomal storage disease gene, is functionally coupled to a regulatory element to obtain expression and secretion of the product (e.g. the lysosomal storage disease protein). According to the invention, useful regulatory elements include promoters, signal peptides, and other elements that are responsible for expression and/or secretion of an expression product such as a lysosomal storage disease protein.

[0070] In a preferred embodiment of the invention, a high level promoter is used to transcribe the gene product of interest.

[0071] In a preferred embodiment of the invention, the sequences surrounding the start codon are optimized for efficient translation in *Leishmania* and/or other protozoa.

[0072] In an alternative embodiment, a gene fragment encoding the structural protein of interest is inserted into an endogenous gene of the protozoan being used. Accordingly, transcription and or translation is achieved using endogenous regulatory sequences.

[0073] In a further embodiment, multiple copies of the gene of interest are introduced into a protozoan, thereby producing a proportional increase in mRNA and protein levels.

C. Use of a signal sequence

[0074] In general, a signal sequence is used to ensure secretion of an expressed gene product according to methods and compositions of the invention.

[0075] In preferred embodiments of the invention, a useful signal peptide is optimized for entry into the secretory pathway of the protozoan that is being used. Example 7 exemplifies an expression construct with a signal peptide that is optimized for *Leishmania*. In general, a signal peptide from an endogenous gene is useful to secrete a heterologous protein from a protozoan. In a preferred embodiment, the coding sequence of a gene of interest is fused, in frame, to a nucleic acid encoding all or part of an endogenous signal peptide. Accordingly, a preferred signal peptide for expression in a chosen protozoan is a signal peptide from that protozoan. However, signal peptides from other organisms, preferably other protozoa, can be used in chimeric expression constructs of the invention.

[0076] Non-limiting examples of *Leishmania* signal peptides include the GP63, secreted acid phosphatase, 3' nucleotidase, chitinase and cystein proteinase signal peptides. Non-limiting examples of *Trypanosoma* signal peptides include the PARP and VSG signal peptides.

[0077] In an alternative embodiment of the invention, the natural signal sequence of the protein being expressed is used. Examples 2-6 show that the natural signal sequences of human GUS and GLA are effective in *L. mexicana*.

[0078] In a further embodiment, a protein is produced in a protozoan that has been modified or selected to increase activity of its secretory pathway.

IV. Administration of expression product to a patient

A. Methods and compositions for administering a gene product purified from an *in vitro* expression system

[0079] Isolated gene products may be administered to a patient using methods known in the art. For example, an LSD protein may be administered to a patient as described in U.S. Patent No. 5,433,946 the disclosure of which is incorporated herein by reference.

B. Methods and compositions for administering a microorganism expressing a gene product

[0080] According to methods of the invention, an engineered protozoan that expresses a gene product is administered to a patient suffering from lack of the gene product.

[0081] In a preferred embodiment, an engineered protozoan is administered intravenously. In alternative embodiments an engineered protozoan is administered subcutaneously, intramuscularly, intraperitonealy, or intracranially. In another embodiment, an engineered protozoan, preferably a gut resident organism, is administered orally. In a further embodiment, an engineered intracellular protozoan is exposed to the patient's cells in vitro. "Infected" cells are then returned to the patient. For example, an engineered *Leishmania* can be used to infect macrophages from a patient in vitro. The infected macrophages are then returned to the patient.

[0082] The amount of an engineered protozoan to be administered to a patient is influenced by several factors, including the expression level of the gene product, the growth characteristics of the protozoan, and the nature of the gene defect in the patient.

[0083] Typically, for lysosomal storage diseases, the amount of enzyme required to rescue the defect is lower than the amount of enzyme present in a healthy individual. Accordingly, a preferred dosage of an engineered protozoan is one that rescues the disease symptoms in the patient.

[0084] Symptoms can be monitored using methods known in the art. The in vivo expression of a gene product according to the invention can be monitored. An example of an in vivo expression assay is shown in Example 9.

[0085] The invention is illustrated further by the following non-limiting examples.

EXAMPLES

Example 1. Method for cloning lysosomal storage disease enzymes into expression vectors.

[0086] The following non-limiting examples illustrate embodiments of the present invention for producing constructs to express lysosomal storage disease enzymes in protozoa. In the following constructs, each LSD gene was cloned into the unique XbaI or BglII sites of the expression plasmid pIR1SAT (FIG. 1A). Nucleic acid fragments containing each LSD gene were generated by PCR amplification of the coding sequence of the LSD gene from a plasmid template obtained from the ATCC, using appropriately designed oligonucleotides having XbaI or BglII restriction sites or sites with overhangs that are complementary to XbaI or BglII restriction overhangs.

i. GLA expression construct

[0087] A human β-galactosidase A (GLA) gene coding fragment was amplified using the following oligonucleotides based on the known GLA sequence:

GLAXX4 5' CGG TCC CCG GGA CAA TGC AGC TGA GGA AC 3'

GLAXXRV 5' ATA TCT AGA TTA AAG TAA GTC TTT TAA TGA 3'

The amplified coding fragment was cloned directionally between the SmaI and XbaI sites of pIR1SAT. The resulting plasmid, pIRGLA-Xa5, contains the GLA insert in the appropriate orientation (in the same orientation as the upstream *L. major* ribosomal small subunit gene (*L. major* SSU in FIG. 1A) so that transcription of the inserted GLA gene is initiated by transcription in the *L. major* ribosomal small subunit gene). The sequence of the GLA insert was verified.

ii. βGUS expression construct

[0088] A human ß-glucuronidase (βGUS) gene coding fragment was amplified using the following oligonucleotides based on the known βGUS sequence:

MULTGUSB4 5' GGT TCT AGA TCT GGG GAC CGG GAA GCA TGG CCC GG 3'

MULTGUSBRV 5' TAT CTA GAT CTT TAG TGT TCC CTG CTA GAA TAG ATG AC 3'

[0089] The amplified coding fragment was cloned into the XbaI site of pIR1SAT. The resulting plasmid, pIRßGUS-X72, contains the βGUS insert in the appropriate orientation (in the same orientation as the upstream *L. major* ribosomal small subunit gene (*L. major* SSU in FIG. 1A) so that transcription of the inserted βGUS gene is initiated by transcription in the *L. major* ribosomal small subunit gene). The sequence of the βGUS insert was verified.

iii. GBA expression construct

[0090] A human glucocerebrosidase (GBA) gene coding fragment was amplified using the following oligonucleotides based on the known GBA sequence:

[0091] DSTGBARV 5' CAC CTC TGC GTG TCG CCC GCA TCG CCT TCC TCT TCC TCT GCC TCA GAT CTA GAC AGT GAG CTC ACC CTA GGC CT 3'

DSTGBA4 5' CCT GTC CCC GGG GGA TCC ACT AGT TCT AGA GGA TCG GAG GTG TGT GAG ATC TCA AGC CTT TGA GTA GGG TAA GC 3'

[0092] The amplified coding fragment was cloned into the XbaI site of pIR1-SAT. The resulting plasmid, pIRGBA-X58, contains the insert in the appropriate orientation (in the same orientation as the upstream *L. major* ribosomal small subunit gene (*L. major* SSU in FIG. 1A) so that transcription of the inserted GBA gene is initiated by transcription in the *L. major* ribosomal small subunit gene). The sequence of the GBA insert was verified.

iv. Other expression constructs

[0093] Using similar amplification and cloning methods, additional clones were produced, including GLA and GBA expression constructs with the coding sequence inserted into the BglII site of pIR1-SAT, Hexosaminidase A (HexA) expression constructs with the coding sequence inserted into the XbaI or the BglII site of pIR1-SAT,

Hexosaminidase B (HexB) expression constructs with the coding sequence inserted into the XbaI or the BglII site of pIR1-SAT, an expression construct with HexA inserted into the BglII site and HexB inserted into the XbaI site of pIR1-SAT, α-Neuraminidase expression constructs with the coding sequence inserted into the XbaI or the BglII site of pIR1-SAT, and N-aspartyl-β-glucosaminidase expression constructs with the coding sequence inserted into the XbaI or the BglII site of pIR1-SAT.

[0094] Similar expression constructs for all LSD enzymes with known sequences can be generated using techniques known in the art.

Example 2. Method for transfecting Leishmania promastigotes

[0095] *Leishmania* promastigotes were maintained in culture at 26-27°C by serial passage of 10 mL cultures at dilutions of 1:100 in 25cm^3 tissue culture flasks containing complete M199 media described in LeBowitz, J. H. (1994, Transfection experiments with *Leishmania*. Microbes as tools for cell biology. D. G. Russell. San Diego, Academic Press. 45: 65-78). Under these conditions, doubling times for promastigote cultures range from about 8-20 hours and saturation densities range from 1-8 x10^7 cells/mL depending on the particular species and isolate.

[0096] Promastiogotes were transfected as follows. Promastigote preparations were electroporated with either 1 μg of gel purified linear expression construct DNA for genomic integration and expression or with 10 μg plasmid DNA for episomal expression. Selection of Leishmania clonal transfectants was carried out as described in LeBowitz, J. H. (1994, Transfection experiments with *Leishmania*. Microbes as tools for cell biology. D. G. Russell. San Diego, Academic Press. 45: 65-78. The selective antibiotic, nourseothricin was present in selective plates at a concentration of 100 μg/mL and in liquid cultures at 50 μg/mL.

[0097] In order to increase the number of chromosomal inserts, 10 μg of linear DNA may be electroporated into a promastigote preparation in order to induce the integration of multiple gene copies.

Example 3. Methods for assaying lysosomal storage disease enzyme activities in promastigotes

[0098] In the following non-limiting examples, LSD enzymes were assayed in both culture media and cell lysates to monitor the extent of enzyme secretion. An assay for glucose-6-phosphate dehydrogenase (G6PDH), a cytosolic protein, was used as a control for cell partitioning. Samples were performed in triplicate.

i. GLA assay

[0099] GLA levels were measured using the assay described below, adapted from Desnick et al., J. Lab. And Clin. Medicine (1973) 81:157-171.

ii. GBA assay

[0100] GBA levels were measured using the assay described below.

iii. βGUS assay

[0101] βGUS levels were measured using the assay described below, adapted from Wolfe and Sands, Protocols for Gene Transfer in Neuroscience (1996), Ed. Lowenstein and Enquist, Chp. 20.

iv. G6PDH assay

[0102] G6PDH levels were measured using the assay described below, adapted from Heise and Opperdoes, MBP 99(1999) 21-32, and Leishmania Lysates: LeBowitz, Trends Cell Biol, (1994) 45 p.63-76.

v. Assay reagents

[0103] The stock solutions used in the assays are as follows. 100X GLA Substrate is 5 mL of absolute EtOH added to a 250 mg vial of 4-methylumbelliferyl β-D-GAL (4-MU-β-D GAL) (the substrate does not dissolve, and it is stored in a vial in a –20°C dessicator with the cap wrapped in parafilm). 100X GBA Substrate is 690 mg 4-MU-β-D glucopyranoside (glucoside) (MUBGlc) added to 8 mL absolute EtOH (again

the substrate does not dissolve, and it is stored in a vial in a –20°C dessicator with the cap wrapped in parafilm). 25X GUS Substrate is 4.55 mL absolute ethanol (EtOH) added to a 250 mg vial of 4-MU-β-D glucuronide (MUG) (again the substrate does not dissolve, and it is stored in a vial in a –20°C dessicator with the cap wrapped in parafilm). 100mM triethanolamine, pH 8 is 9.3 g triethanolamine (Sigma T-9534) dissolved in 500 mL water, adjusted to pH 8, and filter-sterilized. 100X $NADP^+$ (50mM) is 766 mg $NADP^+$ (Sigma N-0505) dissolved in 20 mL 100mM triethanolamine, pH 8, and filter-sterilized. 100X D-glucose 6-phosphate (G6P) (500mM) is 2.82 g G6P (Sigma G-7879) dissolved in 20 mL 100mM triethanolamine, pH 8, and filter-sterilized. 10X Cit/Phos Buffer (0.5M citric acid, 1M Na_2PO_4, pH 4.7) is 10.5 g citric acid and 26.8 g Na_2PO_4 dissolved in 67 mL water, pH to 4.7, filled to 100 mL, and filter-sterilized. Other stock solutions used are 1M Sodium Acetate, pH 4.8 (pH with acetic acid), Triton X-100, Hank's Balanced Salt Solution (HBSS) (Gibco), and 1M $MgCl_2$. The protease inhibitor stock solutions (See LeBowitz Trends Cell Biol, (1994) 45 p.63-76) are 100 mg/mL benzamidine in EtOH, 5 mg/mL leupeptin, 100 mg/mL 1,10 phenanthroline, 0.5 M EDTA, 10 mg/mL soybean trypsin inhibitor, 10 mg/mL BSA (molecular biology grade), and 100 mg/mL PMSF in methanol.

[0104] The working solutions used in the assays are as follows. Cell Lysis Mix is made fresh with the following components (per 1 mL) 980 µL of HBSS, 1.5 µL of Benzamidine, 4 µL of Leupeptin, 2 µL of Phenanthroline, 2 µL of 0.5 M EDTA, 5 µL Trypsin inhibitor, 5 µL of BSA, 1.7 µL of PMSF, and 10 µL of Triton X-100. The Stop Solution is 10.6 g Na_2CO_3, 12.01 g Glycine, H_2O to 500 mL, with the pH adjusted to 10.5, and filter-sterilized. GLA reaction mix is (per 1mL) 890 µL H_2O, 100 µL 10X cit/phos buffer, and 10 µL 100X GLA substrate. GBA reaction mix is (per 1mL) 890 µL H_2O, 100 µL 10X cit/phos buffer, and 10 µL 100X GBA substrate. GUS reaction mix is (per 1mL) 860 µL H_2O, 100 µL 1M NaAc, and 40 µL 25X GUS substrate. G6PDH reaction mix is (per 100mL), 98 mL 100 mM triethanol amine pH 8, 50 µL 1 M $MgCl_2$, 1 mL 100X □□$NADP^+$, and 1 mL 100X G6P.

vi. Assay protocol

[0105] Assays to measure levels of GLA, GBA, GUS and G6PDH were performed using the following protocol.

1) Number 1.5 mL eppendorf tubes, Coulter cups, spectrophotometer and fluoremeter cuvettes (both types of cuvettes should be labeled in tripticate). Fill Coulter cups with 10 mL electrolyte solution.
2) Transfer 1 mL *Leishmania* cells into eppendorf tubes and 0.1 mL cells into Coulter cups containing electrolyte solution. Obtain cell counts when time is available (during later incubation steps). Note: samples do not have to be in triplicate at this point (see steps 6-7)
3) Harvest cells in eppendorf tubes at 3K rpm for 5 min. Remove ~500 mL media (without disturbing cell pellet) from each tube and transfer to a new eppendorf tube. Aspirate off remaining media.
4) Wash each cell pellet in 500 mL HBSS. Spin as above, aspirate HBSS, and suspend cell pellets in 100 µL lysis buffer.
5) Pulse-vortex cells and incubate on ice for 15 minutes. Spin lysates at full-speed for 15 min to pellet cell debris.
6) Transfer 150 µL of the appropriate reaction mix (GLA, GBA or GUS) to fluorimeter cuvettes (in triplicate) and 1 mL of the G6PDH reaction mix to spectrophotometer cuvettes (in triplicate).
7) Transfer 38 µL of media into GLA, GBA or GUS cuvettes and G6PDH cuvettes. Transfer 3.8 µL of cell lysates into GLA,GBA or GUS cuvettes and G6PDH cuvettes.
8) Cover GLA and GUS cuvettes with parafilm (do not cover cuvette box with lid) and incubate 1 hour in 37°C incubator. Incubate G6PDH cuvettes at room temperature overnight.
9) Stop GLA, GBA and GUS reactions by adding 2 mL of glycine/carbonate stop solution.
10) Read fluorescence on Versafluor fluorimeter.

vii. Results

[0106] Expression of ßGUS and GLA was shown in both *L. mexicana* and in *L. major ΔDHFR-TS*).

[0107] FIGs 2 and 3 show that GLA is secreted from *L. mexicana* promastigotes into the culture medium. FIGs 4 and 5 show that ßGUS is secreted from *L. mexicana* promastigotes into the culture medium. FIGs 8 and 9 show that GLA and ßGUS were expressed from many independently isolated *L. mexicana* cell lines. FIGs 10A and 10B show that GLA and ßGUS accumulate in *L. mexicana* culture supernatants over time.

[0108] Expression of GBA, HexA, HexB, and HexS was also shown in *Leishmania* as described in Example 12. Other examples of preferred constructs include neuraminidase (NEU1) and aspartylglucosaminidase (AGU) expression constructs.

Example 4. Methods for assaying lysosomal storage disease enzyme activities in amastigotes

[0109] Promastigotes of *L. mexicana* were converted to amastigotes by pelleting ~ 0.5 mL of promastigote culture, removing the M199 media, then resuspending the cells in 5 mL of modified UM54 medium (Moore, Santrich et al. 1996). Flasks were incubated horizontally at 32-33°C. Amastigotes are passed by 1:10 dilution into pre-warmed UM54 every 3-4 days.

[0110] Assays for LSD enzyme expression were performed on amastigote cultures derived from the promastigote cell lines described above. FIGs 6 and 7 show that GLA and ßGUS are secreted from *L. mexicana* amastigotes into the culture medium.

Example 5. Gene Product Expression in serum free media

[0111] *Leishmania tarentolae* expressing a lysosomal storage disease enzyme is grown in medium in the absence of serum in HyQ™ FXS Serum Free X-Insect (HyClone). The culture is grown to high density, and the enzyme is purified from the culture supernatant.

[0112] Expression product can also be isolated from serum free media using other protozoa, including other *Leishmania* species. In general, the expression strain is grown in medium with serum, diluted into serum free medium, and allowed to grow for several generations, preferably 2-5 generations, before the expression product is isolated. For example, production of secreted recombinant LSD proteins can be isolated from *Leishmania mexicana* promastigotes that are cultured initially in 50 mL 1X M199 medium in a 75 cm2 flask at 27° C. When the cell density reaches 1-3x 10^7/mL, the culture is used to inoculate 1.2 L of M199 media. When the density of this culture reaches about 5x10^6/mL, the cells were harvested by centrifugation, resuspended in 180 mL of the supernatant and used to inoculate 12 L of "Zima" medium in a 16 L spinner flask. The initial cell density of this culture is typically about 5x 10^5 /mL. This culture is expanded to a cell density of about 1.0 – 1.7 x 10e^7 cells/mL. When this cell density is reached, the cells are separated from the culture medium by centrifugation and the supernatant is filtered at 4oC through a 0.2 μ filter to remove residual promastigotes. The filtered media was concentrated from 12.0 L to 500 mL using a tangential flow filtration device (MILLIPORE Prep/Scale-TFF cartridge).

[0113] Preferred growth media for this method are M199 and "Zima" growth media. However, other serum containing and serum free media are also useful. M199 growth media is as follows: (1L batch) = 200 mL 5X M199 (with phenol pH indicator) mixed at 5X + 637 mL H_2O, 50.0 mL FBS, 50.0 mL EF, 20.0 mL of 50g/mL SAT, 2.0 mL of 0.25% hemin in 50% triethanolamine , 10 mL of 10mM adenine in 50mM Hepes pH 7.5, 40.0 mL of 1M Hepes pH 7.5, 1mL of 0.1% biotin in 95% ethanol, 10.0 mL of penicillin/streptomycin. All serums used are inactivated by heat. The final volume = 1 L and is filter sterilized. "Zima" modified M199 media is as follows: (20.0 L batch) = 217.8g M199 powder (–)phenol red + 7.0g sodium bicarbonate, 200.0 mL of 10mM adenine in 50mM Hepes pH 7.5, 800.0 mL 0f Hepes free acid pH 7.5, 20.0 mL 0.1% biotin in 95% ethanol, 200.0 mL penicillin/streptomycin, 2780.0 mL H20 Final volume = 20.0 L and is filter sterilized.

Example 6. *Leishmania* expression vectors

[0114] The following non-limiting example is illustrative of methods and compositions for increasing expression and secretion of gene products in protozoa. Useful secretion of an LSD enzyme from *Leishmania* will be obtained using an expression vector that facilitates the construction and expression of hybrid proteins containing a signal peptide derived from a *Leishmania* protein, fused to the portion of the LSD gene that encodes the mature LSD enzyme. Useful expression will be obtained using a signal peptide that is optimized for maximal expression. Useful expression will also be obtained using a vector in which sequences 5' to the start codon are optimized for maximal translation. Translation of a gene coding for an LSD enzyme will be maximized in *Leishmania* by altering the codon usage of the LSD gene to reflect the codon bias of *Leishmania*. Useful expression of LSD genes will be driven by including a bacteriophage RNA polymerases (such as T-7 RNA polymerase) in an expression vector. Examples of different expression systems for optimizing gene, and particularly LSD gene expression and/or secretion are provided in Example 13.

Example 7. Signal Peptide Increased Translation and Secretion Plasmid

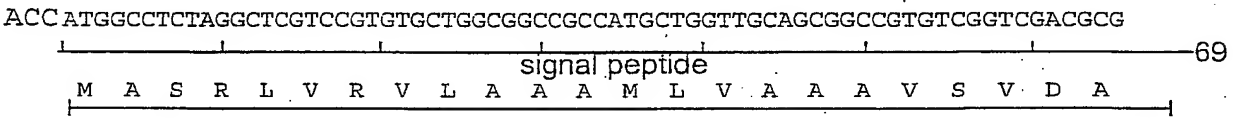
[0115] Plasmid pSPITS (Signal Peptide Increased Translation & Secretion) is shown in FIG. 1B. Sequences between the unique NarI and XbaI sites of pIR1-SAT are replaced with sequences from the *L. major* gp63 locus containing the 5' intergenic region, including a putative splice acceptor site and the 5' end of the polypeptide containing the signal peptide. This cassette is amplified from *L. major* genomic DNA using oligos:

GP63 5' 5' CAGATCGATCTCGAGGGTGCTGTCCCCCCTCGCTGCGGCGT 3'
GP63 3' 5' AGGTCTAGATGCCCACGCGGCCGCGGTGCCGACA 3'

[0116] The 5' oligo has a ClaI site that is compatible with the NarI site in the vector and the 3' oligo has an XbaI site. Gene cassettes are easily cloned into the resulting plasmid, between the unique NotI and XbaI sites. Appropriately designed oligonucleotides are used to amplify a gene cassette by PCR, the gene cassette is cloned

into pSPITS and the resulting plasmid expresses a chimeric gene containing the gp63 signal peptide fused to the polypeptide encoded by the gene cassette.

[0117] An alternative construct is made by inserting an oligonucleotide encoding the signal peptide of the *L. mexicana* secreted acid phosphatase, SAP-1, into the XbaI site of a modified pIR1-SAT in which the single SalI site has been removed. Appropriately designed oligonucleotides are used to amplify a gene cassette by PCR, and the gene cassette is cloned into the plasmid. The resulting plasmid expresses a chimeric gene containing the SAP signal peptide fused to the polypeptide encoded by the gene cassette.



SAP-1 signal peptide.

[0118] To conform the 3 nucleotides 5' to the start codon of ßGUS and GLA to desirable sequences for optimal translation in *Leishmania* expression constructs were made using the following oligonucleotides to modify the 5' region. In each case, the relevant oligonucleotides used to amplify the gene cassettes are listed below with the altered trinucleotide sequence highlighted in bold. The first oligonucleotide of each set has the endogenous sequence.

Oligonucleotides for GLA 5' modification:

GLAXACA 5' CCACTCTAGAACAATGCAGCTGAGGAACCCAGAACTA 37

GLAXACC 5' CCACTCTAGA**ACC**ATGCAGCTGAGGAACCCAGAACTA 37

GLAXATC 5' CCACTCTAGA**ATC**ATGCAGCTGAGGAACCCAGAACTA 37

GLAXCCC 5' CCACTCTAGA**CCC**ATGCAGCTGAGGAACCCAGAACTA 37

Oligonucleotides for GUS 5' modification:

GUSXAGC 5' CCACTCTAGAAGCATGGCCCGGGGGTCGGCGGTTGCC 37

GUSXACC 5' CCACTCTAGAACCATGGCCCGGGGGTCGGCGGTTGCC 37

GUSXATC 5' CCACTCTAGAATCATGGCCCGGGGGTCGGCGGTTGCC 37

Example 8. Vectors for tagging expression products

[0119] The following non-limiting example illustrates the modification of a protozoan expression system to tag the expressed gene product.

[0120] Modified pIR1-SAT vectors designed to create a C-terminal epitope tagged expression product were constructed. The constructs have a VSV tag (mab available from Roche) inserted into either the XbaI site or the BglII site of pIR1SAT.

[0121] Oligonucleotides for making the tag constructs are VSVB1 and VSVB2 for the BglII site, and VSVX1 and VSVX2 for the XbaI site. For both modifications there are unique XhoI and Asp718 sites that permit appropriately amplified gene cassettes to be ligated in frame with the tag cassette.

[0122] Gla and Gus cassettes were cloned into the XbaI tag vector using each of the 5' modified nucleotide sequences.

Oligonucleotides for the BglII tag site:

GATCTCGGGTACCGCTCGAGTACACGGATATCGAGATGAACCGCCTGGGCAA GTGATCGAT VSVB1

GATCATCGATCACTTGCCCAGGCGGTTCATCTCGATATCCGTGTACTCGAGCG GTACCCGA VSVB2

Oligonucleotides for the XbaI tag site:

CTAGACGGGTACCGCTCGAGTACACGGATATCGAGATGAACCGCCTGGGCAA GTGATCGATA VSVX1

CTAGTATCGATCACTTGCCCAGGCGGTTCATCTCGATATCCGTGTACTCGAGC GGTACCCGT VSVX2

Example 9. *In vivo* expression assays

[0123] *In vivo* expression of an LSD protein is assayed using an enzyme-based, a histochemical, or an immunological method, or a combination of the above, according to methods known in the art.

[0124] According to the invention, to demonstrate that LSD proteins secreted from *Leishmania* contain carbohydrate with terminal mannose residues, recombinant ß-glucuronidase from *Leishmania mexicana* containing plasmsid pXSAP0-GUS was grown in M199 culture medium with a small amount of serum proteins. When the culture reached a density of > 1.0×10^7 promastigotes/mL the *L. mexicana* were removed by centrifugation, 10 min at 500 x g. The harvested culture medium was passed through a 0.2 µm filter to remove particulates before being loaded directly onto a Concanavalin A (ConA)-agarose column (4% cross-linked beaded agarose, Sigma). The ConA-agarose column was pretreated with 1 M NaCl, 20 mM Tris pH 7.4, 5 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$ and then equilibrated with 5 volumes of column buffer (20 mM Tris pH 7.4, 1 mM $CaCl_2$, and 1 mM $MnCl_2$). A total of 179,800 units (nmol/hr) of GUS activity (in 2 L) in culture medium was loaded onto a 22 mL ConA agarose column. No activity was detectable in the flow through or wash. The GUS activity was eluted with column buffer containing 200 mM methyl mannopyranoside. Eluted fractions containing the activity peak were pooled and concentrated: 143900 units of GUS activity were recovered from the column (80% recovery of activity loaded onto the column). This demonstrates that the recombinant ß-GUS secreted from *L. mexicana* did possess carbohydrate with terminal mannose residues and further points out the potential for using the interaction of mannose with ConA as the basis for an affinity purification step.the presence of high mannose carbohydrate can serve as the basis of an affinity step in the purification of recombinant LSD proteins using lectin affinity chromatography.

Example 10. Mannose or mannose-6-phosphate Mediated Internalization of lysosomal Storage Disease Proteins

[0125] In order for LSD proteins produced by stably transfected protozoa to be an effective therapeutic agent for lysosomal storage diseases, the enzyme must be internalized by the affected cells and transported to the lysosome. In most cases, this internalization is mediated by the binding of lysosomal enzymes to mannose-6-phosphate (M6P) receptors, which are expressed on the cell surface of most cell types and deliver the enzyme to the lysosome via the endocytic pathway. The M6P receptor is ubiquitously expressed; most somatic cells express it to some extent. The mannose receptor, which is specific for exposed mannose residues on glycoproteins, is less prevalent. The latter receptors are generally found only on macrophage and macrophage-like cells. The mannose receptor provides an essential means of entry for glucocerebrosidase into cells of the macrophage lineage where it can exert a therapeutic effect.

[0126] In order to demonstrate M6P-mediated internalization of lysosomal enzymes, skin fibroblasts from disease patients (NIGMS Human Genetic Mutant Cell Repository) will be cultured overnight in the presence of increasing concentrations of purified enzyme of the invention. Some of the samples will contain 5 mM soluble M6P, which competitively inhibits binding to, and as a result, internalization by, the mannose-6 phosphate receptor. Other samples will contain 30 μg/mL mannan, which inhibits binding to, and as a result, internalization by, the mannose receptor. Following incubation, the cells will be washed and harvested by scraping into lysis buffer (10 mM Tris, pH 7.2, 100 mM NaCl, 5 mM EDTA, 2 mM Pefabloc.TM. (Roche) and 1% NP-40). The lysed samples will then be assayed for protein concentration and enzyme activity. The results will be expressed as units of enzyme activity/mg cell protein. It is expected that enzymes internalized via the M6P receptor will display a significant amount of cell associated activity that can be competed by the addition of M6P to the media but not by the addition of mannan.

[0127] The mouse macrophage-like cell line (J774.E) which bears mannose receptors but few if any mannose 6-phosphate receptors can be used to determine whether purified glucocerebrosidase of the invention is internalized via the

mannose receptor (Diment et al., J. Leukocyte Biol.42:485-490, 1987). J774.E cells will be cultured overnight in the presence of increasing concentrations of glucocerebrosidase. Selected samples will contain 2 mM M6P, and others will contain 100 µg/mL mannan. The cells will be washed and harvested as described above, and the total protein and glucocerebrosidase activity of each sample will be determined. It is predicted that M6P will not inhibit the uptake of glucocerebrosidase by these cells, while mannan decreases the accumulated glucocerebrosidase significantly.

Example 11. Mannose Mediated Internalization of Glucocerebrosidase

[0128] In order for glucocerebrosidase produced by stably transfected protozoa to be an effective therapeutic agent for Gaucher's disease, the enzyme must be internalized by cells of the macrophage lineage and transported to the lysosome. This internalization is mediated by the binding of glucocerebrosidase to the mannose receptor, which is specific for exposed mannose residues on glycoproteins. The mannose receptors are generally found only on macrophage and macrophage-like cells. The mannose receptor provides a means of entry for glucocerebrosidase into cells of the macrophage lineage where they can exert a therapeutic effect.

[0129] The mouse macrophage-like cell line (J774.E) which bears mannose receptors but few if any mannose 6-phosphate receptors can be used to determine whether purified glucocerebrosidase of the invention is internalized via the mannose receptor (Diment et al., J. Leukocyte Biol.42:485-490, 1987). J774.E cells will be cultured overnight in the presence of increasing concentrations of glucocerebrosidase. Some of the samples will will contain 2 mM M6P, which competitively inhibits binding to, and as a result, internalization by, the mannose-6 phosphate receptor. Other samples will contain 100 µg/mL mannan, which inhibits binding to, and as a result, internalization by, the mannose receptor. Following incubation, the cells will be washed and harvested by scraping into lysis buffer (10 mM Tris, pH 7.2, 100 mM NaCl, 5 mM EDTA, 2 mM Pefabloc.TM. (Roche) and 1% NP-40). The lysed samples will then be assayed for protein concentration and glucocerebrosidase activity. The results will be expressed as units of glucocerebrosidase activity/mg cell protein. It is expected that

glucocerebrosidase internalized via the mannose receptor will display a significant amount of cell associated activity that can be competed by the addition of mannan to the media but not by the addition of M6P.

Example 12. Expression and Secretion of GBA and HexA, HexB, and HexS

[0130] *Leishmania* strains have been engineered to secrete active glucocerebrosidase (GBA) and hexosaminidaseS (HexS), hexosaminidaseB (HexB) and hexosaminidaseA (HexA). Each of these enzymes are secreted in both the promastigote and amastigote stages.

i. GBA Expression

[0131] GBA was successfully expressed using either of two *Leishmania* signal peptides and have also using the longer of two putative naturally occurring GBA signal peptides. Figures 11 and 12 show GBA expression from SAP-GBA constructs described below.

ii. HexA, HexB, and HexS Expression

[0132] HexS protein is a homodimeric protein where each subunit is encoded by the HexA gene. HexB is a homodimeric protein where each subunit is encoded by the HexB gene. HexA protein is a heterodimeric protein where one subunit is encoded by the HexA gene and the other subunit is encoded by the HexB gene. Mutations in the HexA gene are responsible for Tay Sachs disease, but it the pathology is reportedly due to the absence of functional HexA protein and not HexS protein. Sandhoff disease is caused by mutations in the HexB gene, and the pathology is due to lack of functional HexA and HexB. Accordingly, there is a need for methods for expressing, isolating, and delivering functional hexosaminidase enzymes.

[0133] Expression cassettes were made in which the HexA or HexB genes were separately expressed. In addition, constructs were made to simultaneously coexpress the HexA and HexB genes. Two substrates were used to assay the Hex

proteins (see Figures 13 and 14). MUG (4-methylumbelliferyl-GlcNAc) is hydrolyzed by HexA, HexB and HexS proteins. MUGS (4-methylumbelliferyl-GlcNAc-6-SO_4) is hydrolyzed by HexA and HexS only.

Example 13. Expression and Secretion Systems

[0134] To illustrate the general applicability of the invention, three alternative vectors were made to permit fusion of different *Leishmania* signal peptides with mature LSD polypeptides. The first vector uses the signal peptide from SAP (secreted acid phosphatase) and was constructed as described herein. The other two vectors designated pSPITS-1 (referred to as pSPITS in Example 1) and pSPITS-3 contain the gp63 signal peptide and either about 280 or 460 bp of 5' flanking sequence from gp63 cloned into pIR1-SAT as previously herein.

[0135] Each of the GLA, ß-GUS, and GBA cassettes have been inserted into the signal peptide vectors in three ways creating chimeric genes in which the mature gene cassette is fused either at the site of signal peptide cleavage, 1 amino acid residue downstream from the site of cleavage, or 5 amino acids downstream from the site of cleavage. In the latter two cases, the resultant chimeric LSD proteins would have 1 or 5 amino acids at the amino terminus encoded by the *Leishmania* gene that provided the signal peptide.

[0136] Exemplary expression strains described herein follow the following nomenclature. *Leishmania* strains are named with a number and further by denoting the plasmid used for transfection. All plasmids are derived from pIR1-SAT. Plasmids with gene cassettes encoding ß-Glucuronidase contain the name GUS; those with glucocerebrosidase contain GBA in the name; those with a-Galactosidase A contain GLA in the name. Vectors in which the *Leishmania* secreted acid phosphatase signal peptide is fused to the mature LSD gene cassette contain SAP in the name; vectors using the gp63 signal peptide and intergenic region have either pSPITS1 or pSPITS3 in the name depending on the size of the gp63 intergenic region employed. SAP and pSPITS constructs have a further number designation that indicates the site of fusion of the signal

peptide with the LSD gene. For example, in the name pSPITS3-GUS0-93 the '0' after GUS refers to the fact that the fusion is at position 0 relative to the gp63 cleavage site. In pSPITS3-GUS5-100, the '5' refers to the fact that 5 residues downstream of the gp63 cleavage site were included in the signal peptide cassette fused to mature GUS. The same applies to SAP constructs except that the number can occur either after the SAP or after the gene name. For example in the names pSAP-GUS0-21 and pGLA-SAP0-1, the '0' in each case refers to the fusion of the signal peptide to the LSD cassette at position 0 relative to the cleavage site of the SAP signal peptide. Constructs with an 'X' in the name, refer to the cassette being inserted between the XmaI and XbaI sites in the vector.

[0137] Preferred expression constructs are based on combinations of regulatory elements disclosed herein. Preferred expression construct combinations for GBA include those on the following plasmids: pGBA-SAP0, pGBA-SAP1, pGBA-SAP5, pSPITS1-GBA0, pSPITS1-GBA1, pSPITS3-GBA0, pSPITS3-GBA1, pSPITS3-GBA5, pSAP0-GBA, pXGBA and pXGBA-SAP0. Preferred expression construct combinations for GUS include those on the following plasmids: pSAP-GUS0, pSAP-GUS1, pSAP-GUS5, pSPITS1-GUS0, pSPITS1-GUS1, pSPITS3-GUS0, pSPITS3-GUS1, pSPITS3-GUS5, and pXGUS-SAP0. Preferred expression construct combinations for GLA include those on the following plasmids: pGLA-SAP0, pGLA-SAP1, pGLA-SAP5, pSPITS1-GLA0, and pSPITS3-GLA0. These and other combinations of regulatory elements disclosed herein are useful for the expression and secretion of other LSD enzymes in addition to GBA, GUS, and GLA. Alternative expression construct combinations include pGLA-CCC-XTAG, pGLA-ATC-XTAG, pIR-GLA-CCC, pIR-GLABw, pIR-GLA-Xd, pGLA-ACA-XTAG, pGLA-ACC-XTAG, and pIR-ACC-GLA. Different plasmid, regulatory element and gene combinations produced different levels of expression and secretion. For example, the GBA-SAP1 construct gave the highest activity while the SAP-0 construct was less active. However, there is a point mutation in the GBA-SAP -0 construct that changes a threonine residue to an alanine residue, and this could alter the activity of the protein. In another example, higher levels of ß-glucuronidase and α galactosidase A expression in *Leishmania* were achieved with constructs using the *Leishmania* secreted acid phosphatase (SAP) signal peptide. In another example, recombinant *Leishmania* containing gene cassettes in which the

nucleotides upstream of the start codon of ß-GUS, GLA and GBA have been altered. Some of these alterations reproducibly increase expression levels. As a further example, relative ß-GUS activities with different constructs were also tested. In another example, *Leishmania* that express epitope-tagged versions of GLA, ß-GUS and GBA were tested and displayed activity, and tagged proteins (e.g. the tagged- ß-GUS protein) can be detected by western blot.

[0138] The following tables show expression levels for examples of different expression constructs.

Table 3. Relative ß-GUS activities in promastigotes and amastigotes containing different GUS constructs. The Xba and Bgl constructs refer to the original constructs in which the ß-GUS cassette was cloned into the XbaI or BglII site of pIR1-SAT. ACC, ATC etc refer to the constructs in which the nucleotides 5' of the start codon were altered. XTAG refers to the presence of a carboxy terminal VSV- epitope tag.

	Normalized expression			
	Promastigotes		Amastigotes	
Construct	Media	Cells	Media	Cells
Xba	1.00	0.77	1.51	1.66
Bgl	0.58	0.31	0.92	1.02
ACC	3.58	2.29	4.18	2.10
ATC	2.15	1.80	5.05	3.27
AGC	2.71	1.68	3.58	1.99
ACC-XTAG	0.45	3.34	1.00	5.42
ATC-XTAG	0.78	4.31	0.69	3.86
AGC-XTAG	0.54	3.34	0.82	4.58

The following tables show exemplary expression levels in different *L. mexicana* strains.

Table 4: Examples of ecreted activity from *L. mexicana* promastigotes.

Gene	Strain	U in media/ 10^6 promastigotes	U in media/ 10^6 amastigotes
ß-glucuronidase	499.4 pXGUS-SAP0-2	12.31	NA
Glucocerebrosidase	449.4 pSPITS1-GBA1-53	0.96	4.43
α-Galactosidase A	383.1 pGLA-SAP0-6	2.94	0.56

Table 5: Expression of glucocerebrosidase in *L. mexicana* using different signal peptides.

Signal peptide	Strain	U in media/ 10^6 promastigotes	U in media/ 10^6 amastigotes
Endogenous short	167.1 pIRSATGBAX58	0	0
Endogenous long	513.1 pXGBA-25	0.29	NA
SAP	511.3 pXGBA-SAP0-21	0.38	NA
gp63	445.4 pSPITS1-GBA0-49	1.02	3.72

[0139] In addition, several ß-GUS constructs were tested in the ΔDHFR-TS strain of *L. major Freidlen*. Activities approximately paralleled those observed in *L. mexicana*, as shown in the following table.

Table 6: Expression of selected constructs in *L. mexicana* and *L. major Freidlen* Δdhfr-ts.

Plasmid	*Leishmania Mexicana* strain	U in media/ 10^6 cells	*Δdhfr-ts Leishmania major Freidlen* strain	U in media/ 10^6 cells
PXGUS-SAP0-1	497.3	11.6	534.5	9.21
PSAP-GUS1-25	409.5	6.43	537.2	10.67
PSPITS3-GUS0-93	488.2	2.98	539.5	4.40

CLAIMS

What is claimed is:

1. A method for obtaining a lysosomal storage disease enzyme, comprising:

(a) providing a protozoan that expresses a lysosomal storage disease enzyme;

(b) growing said protozoan in an *in vitro* culture; and,

(c) purifying said enzyme from said culture.

2. A method for delivering a lysosomal storage disease enzyme to a patient, comprising:

(a) providing a protozoan that expresses a lysosomal storage disease enzyme under the control of a regulatory element that drives expression and secretion of said enzyme; and,

(b) administering said protozoan to a patient,

wherein said protozoan persists in said host and delivers said enzyme to said patient.

3. A protozoan engineered to express a lysosomal storage disease enzyme, comprising:

(a) a lysosomal storage disease enzyme coding sequence; and,

(b) a regulatory element driving gene expression functionally coupled to said coding sequence,

wherein transcription from said promoter expresses said lysosomal storage disease enzyme.

4. A method for obtaining a prion free expression protein preparation, comprising

(a) providing a protozoan that is engineered to express a protein;

(b) growing said engineered protozoan in a culture medium that is free of factors derived from a mammalian source; and,

(c) purifying said protein from said protozoan culture.

5. The method of claims 2 or 3, wherein said regulatory element comprises a signal peptide from said protozoan.

6. The method of claim 5, wherein said signal peptide is selected from the group consisting of SAP and gp63 signal peptides.

7. The method of claims 1, 2, or 4, wherein said protozoan is a trypanosomatid.

8. The method of claim 7, wherein said trypanosomatid is a *Leishmania* species.

9. The method of claim 8, wherein said *Leishmania* species is selected from the group consisting of *L. mexicana*, *L. major*, and *L. tarentolae*.

10. The protozoan of claim 3, wherein said protozoan is a trypanosomatid.

11. The protozoan of claim 10, wherein said trypanosomatid is a *Leishmania* species.

12. The protozoan of claim 11, wherein said *Leishmania* species is selected from the group consisting of *L. mexicana*, *L. major*, and *L. tarentolae*.

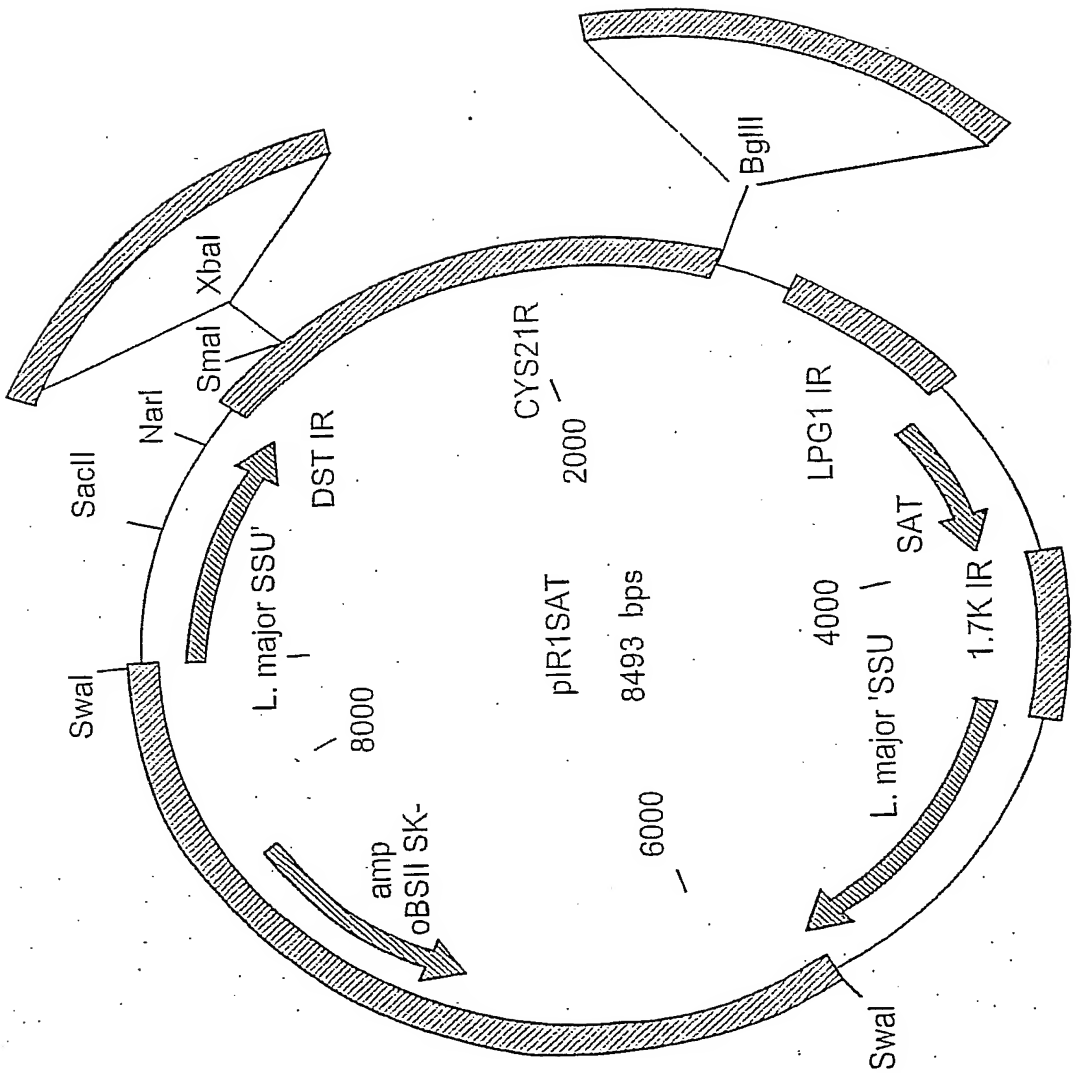


FIG. 1A

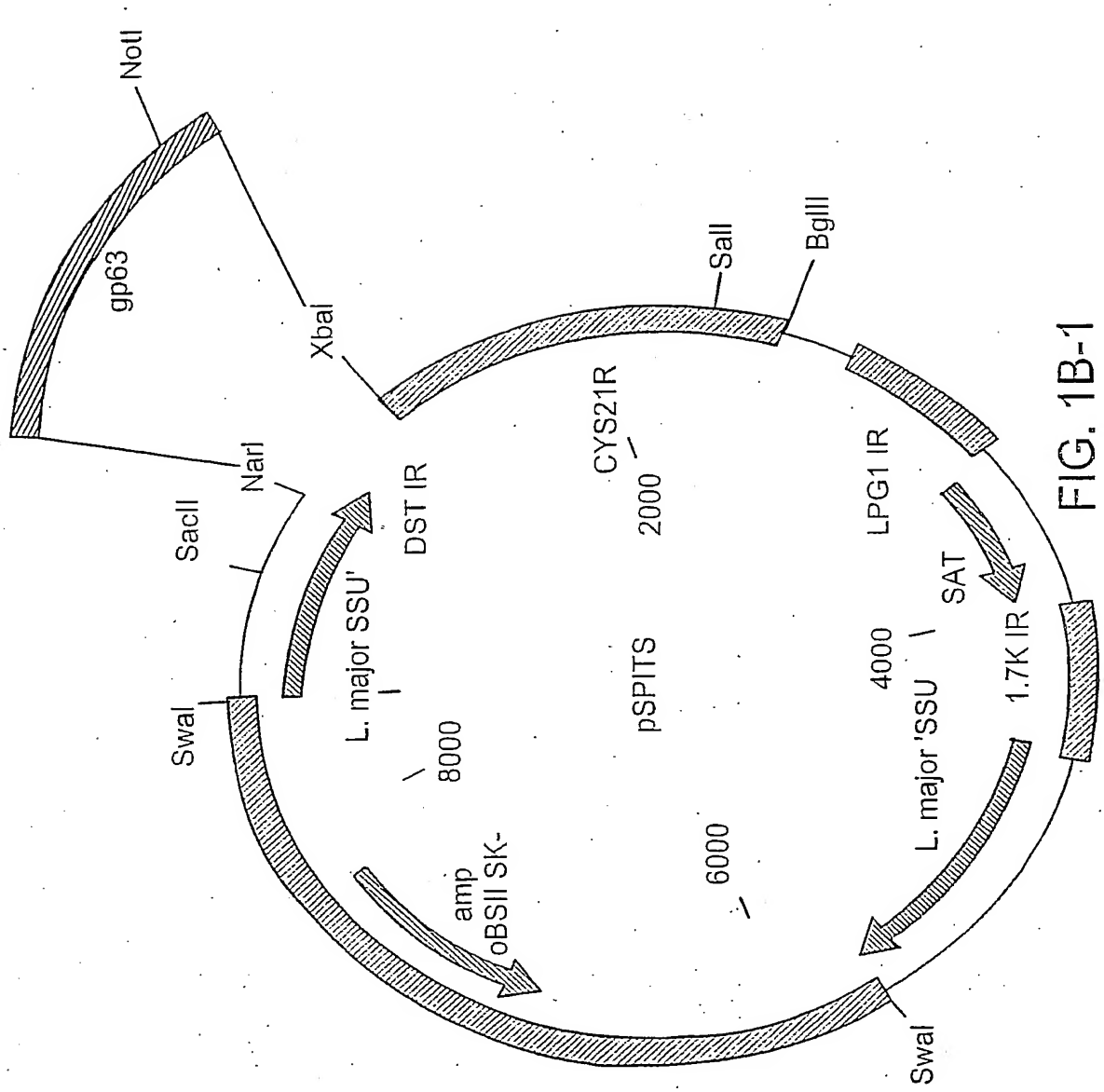


FIG. 1B-1

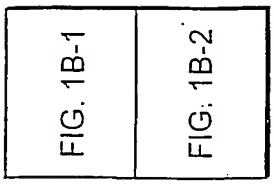


FIG. 1B

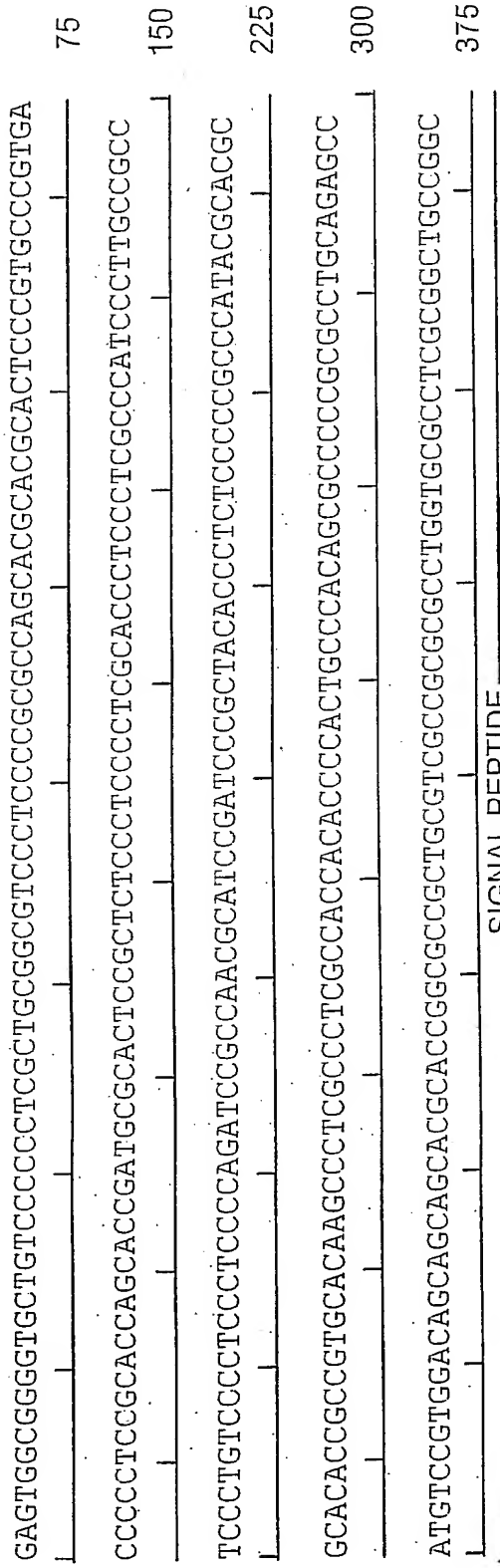


FIG. 1B-2

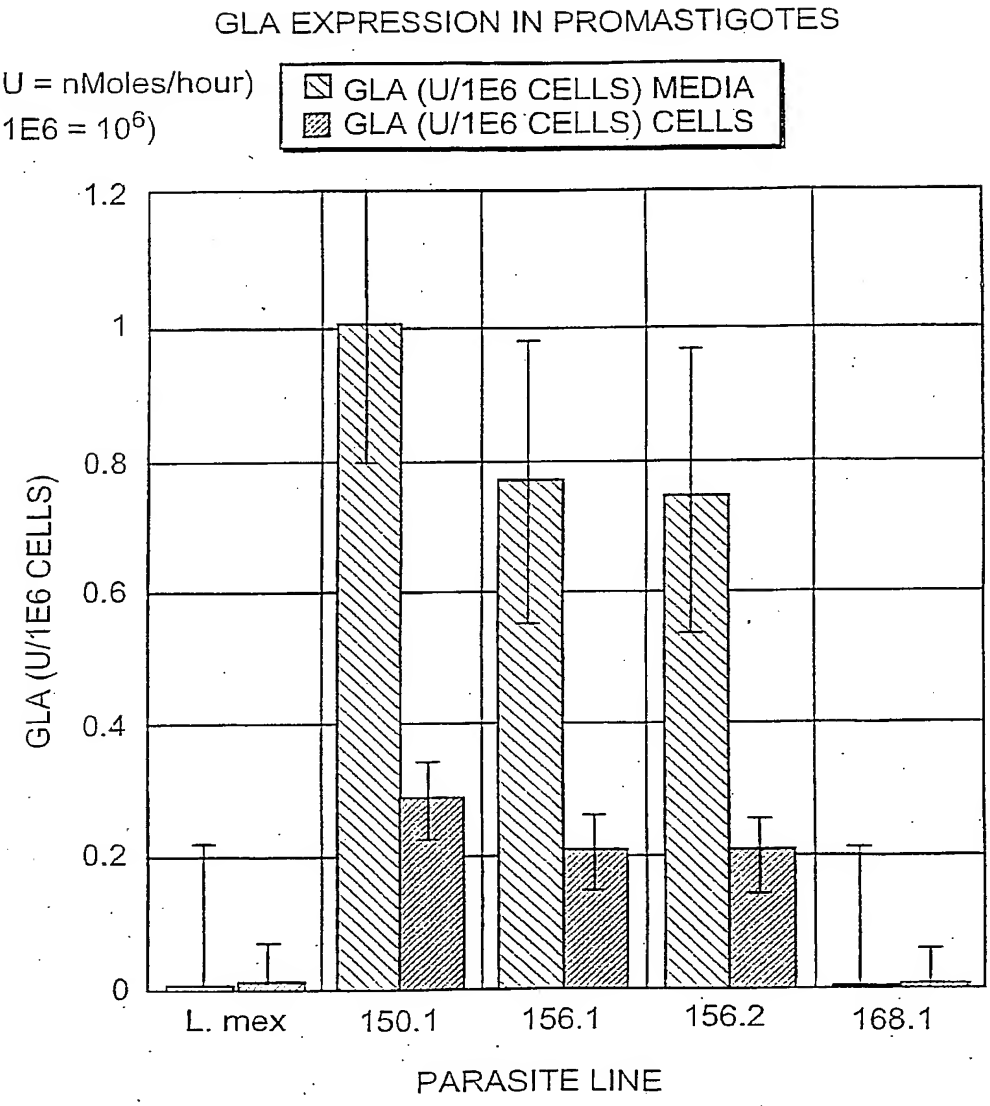


FIG. 2

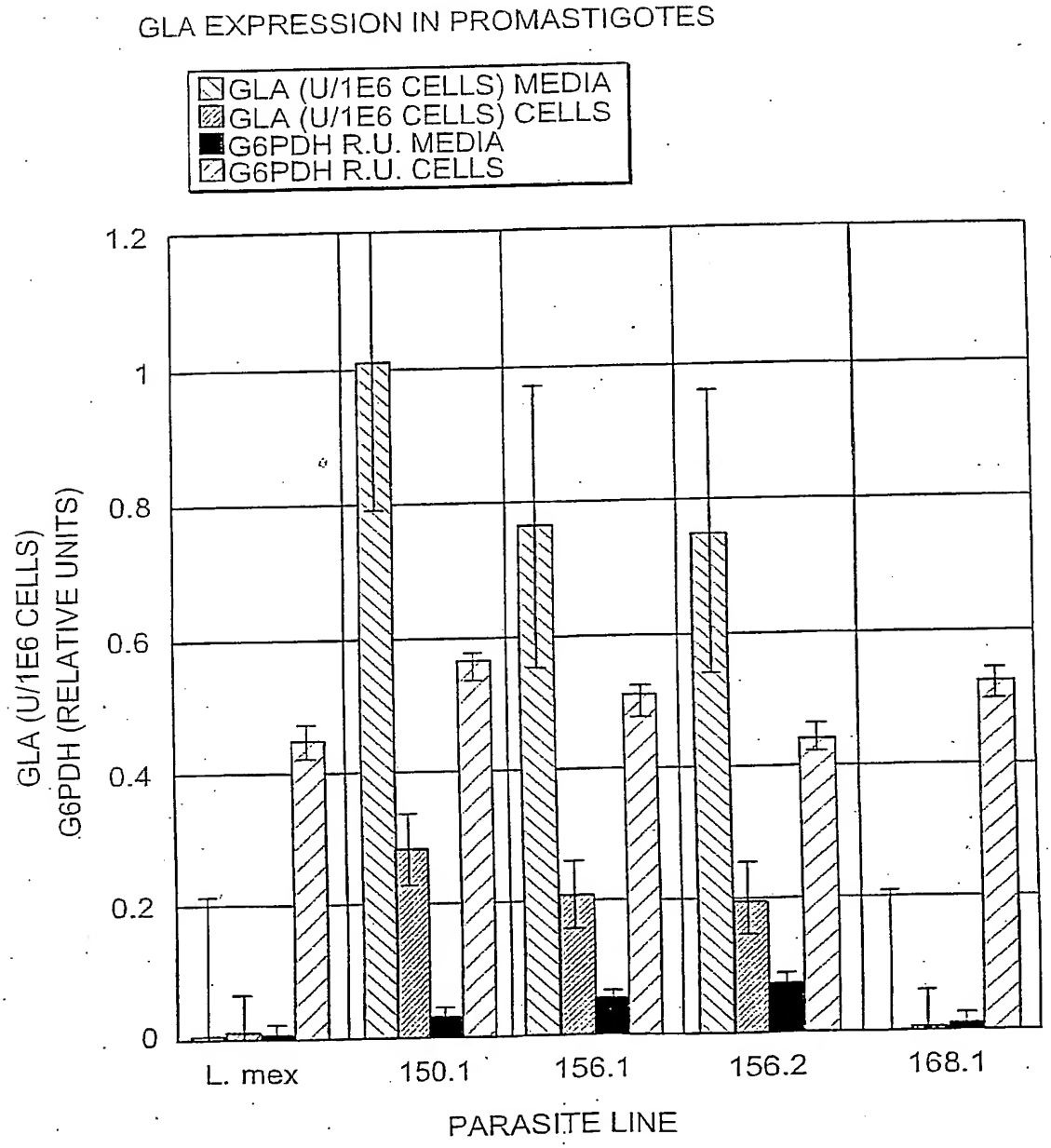


FIG. 3

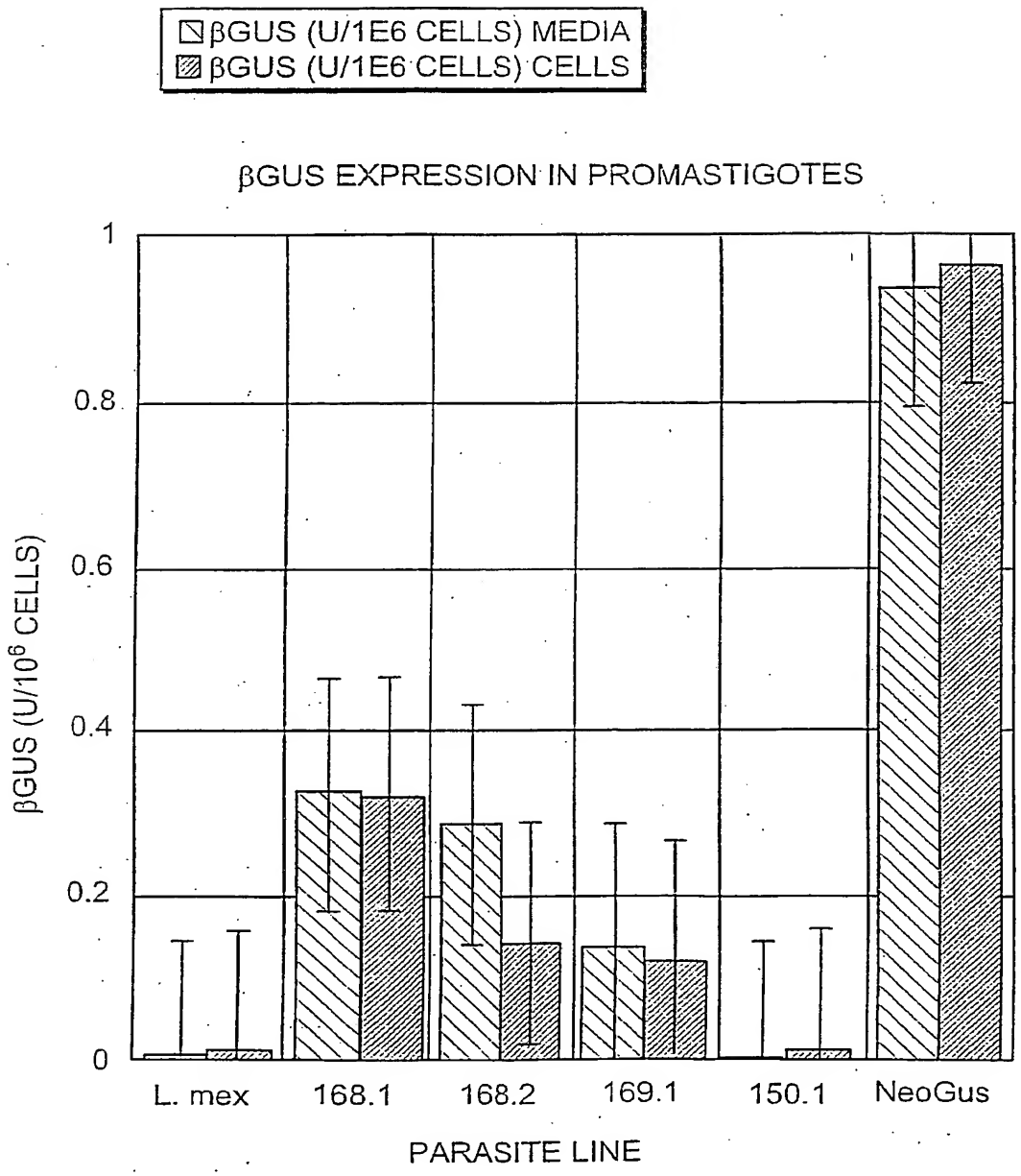


FIG. 4

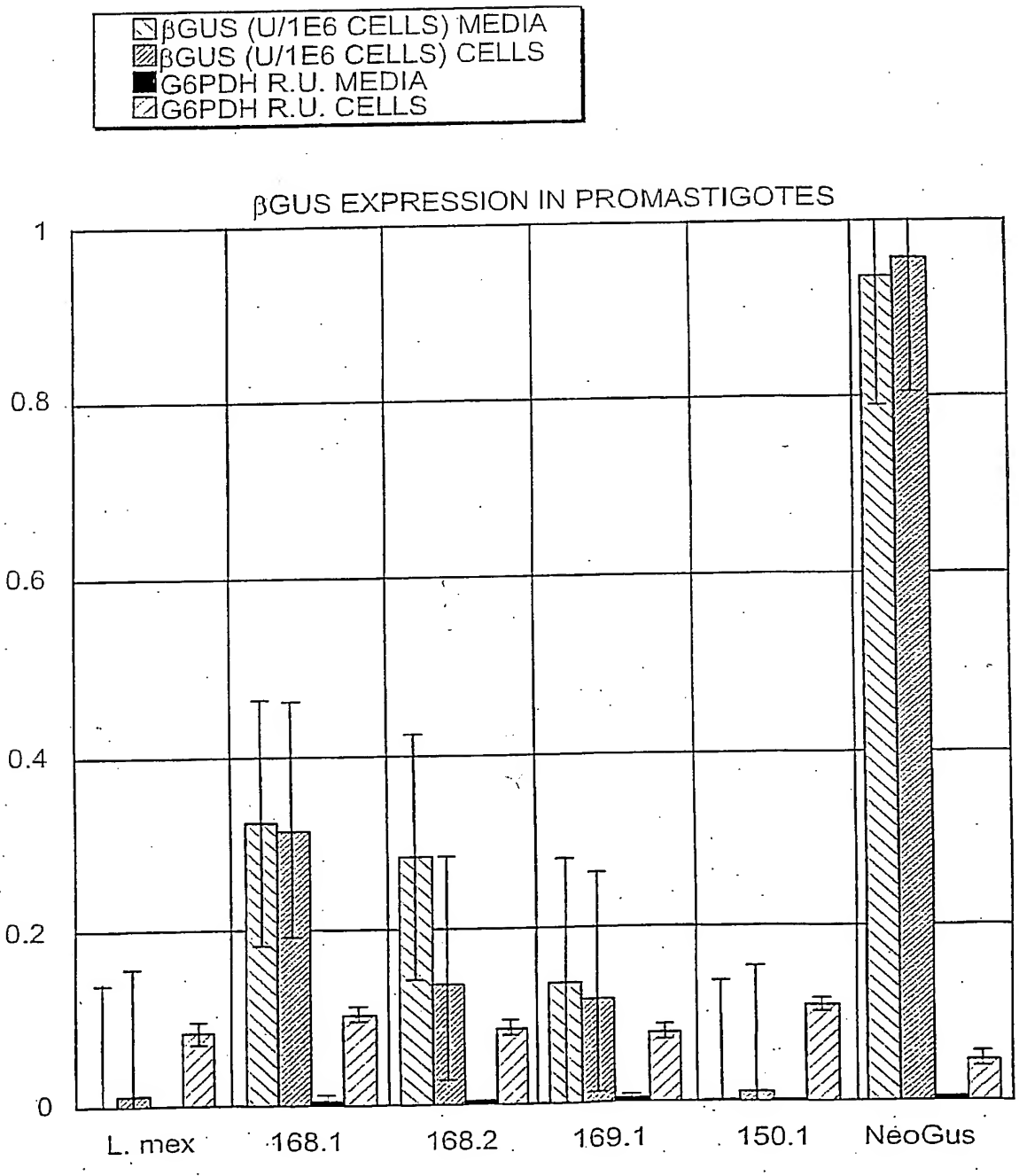


FIG. 5

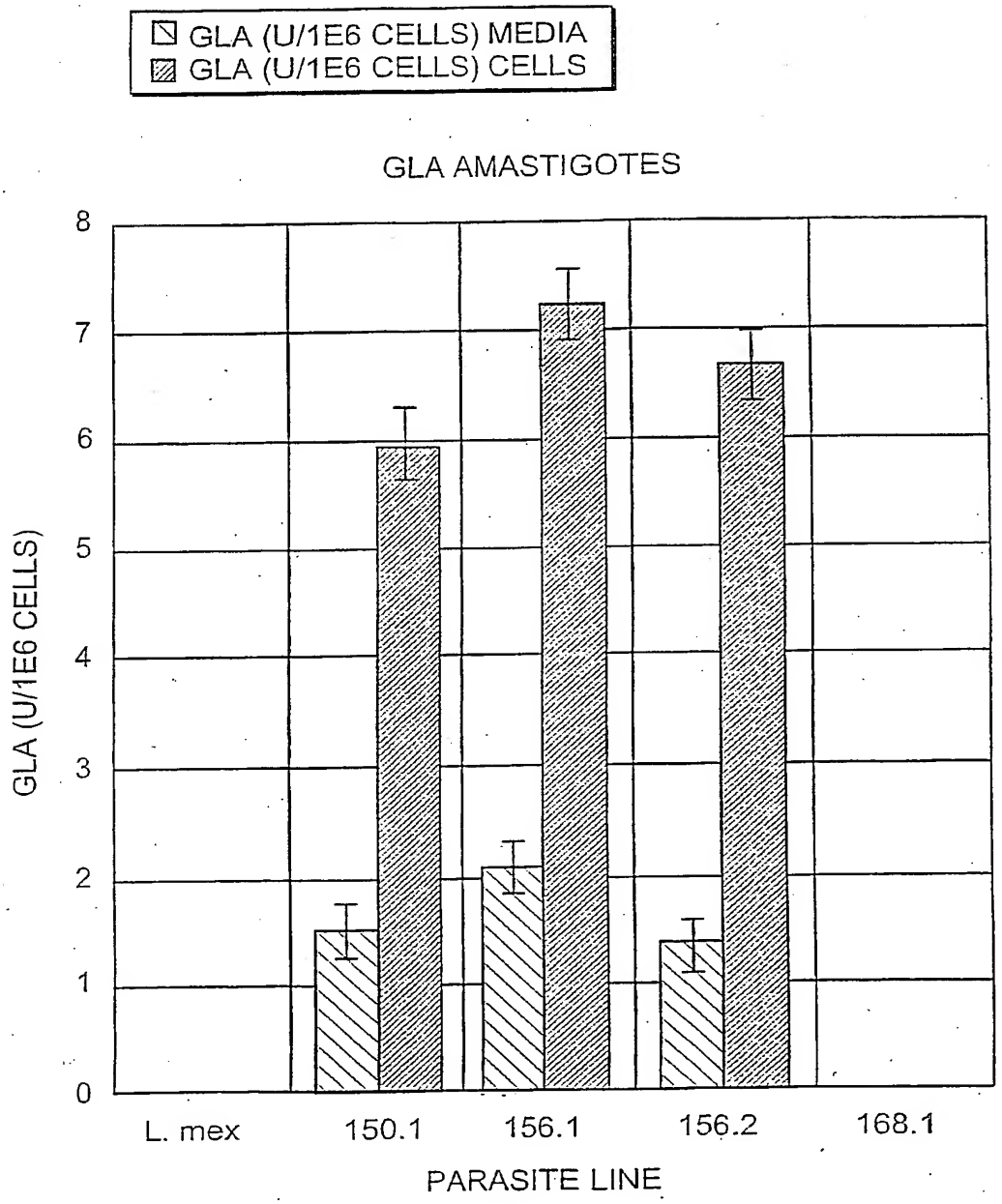


FIG. 6

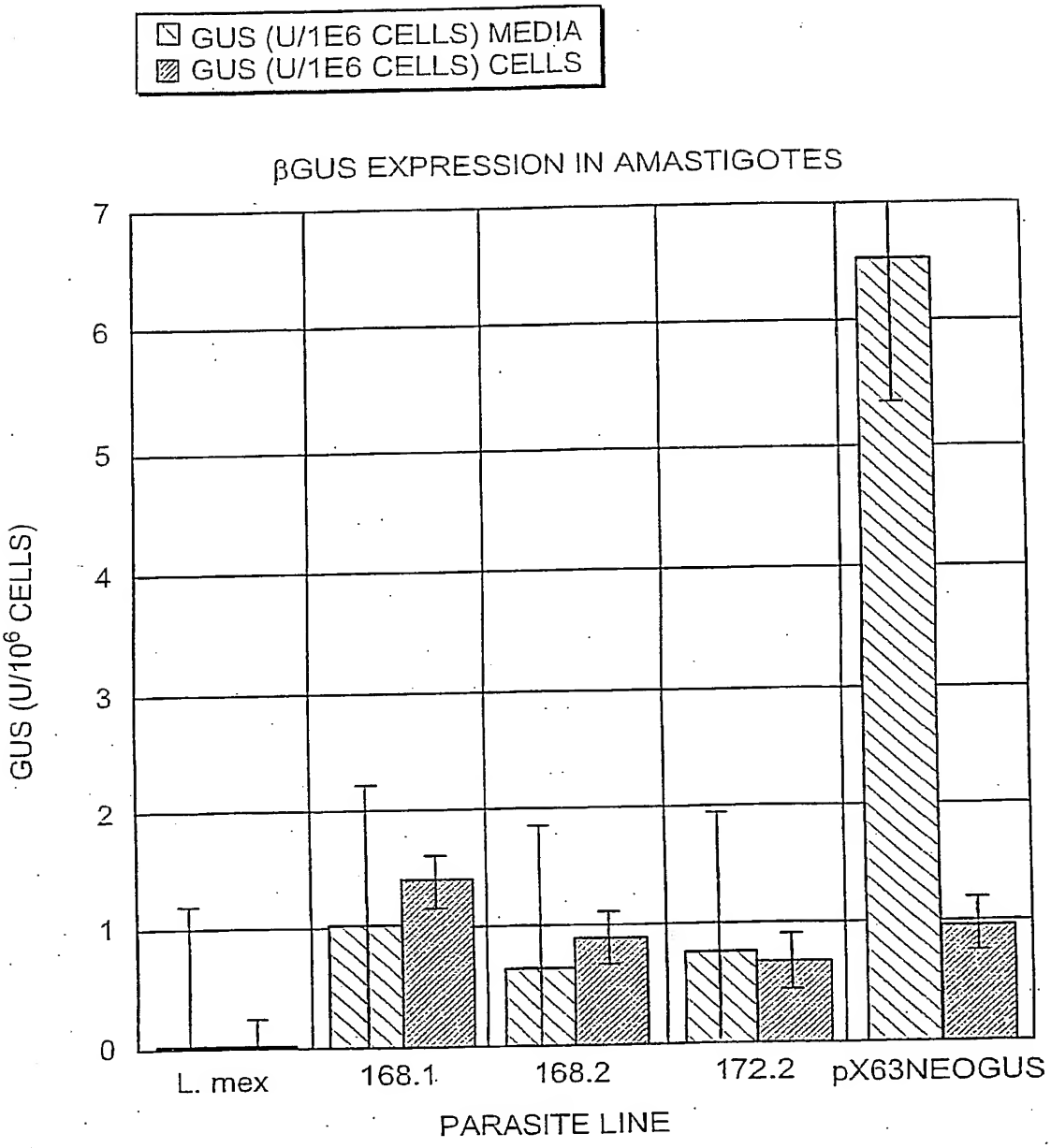


FIG. 7

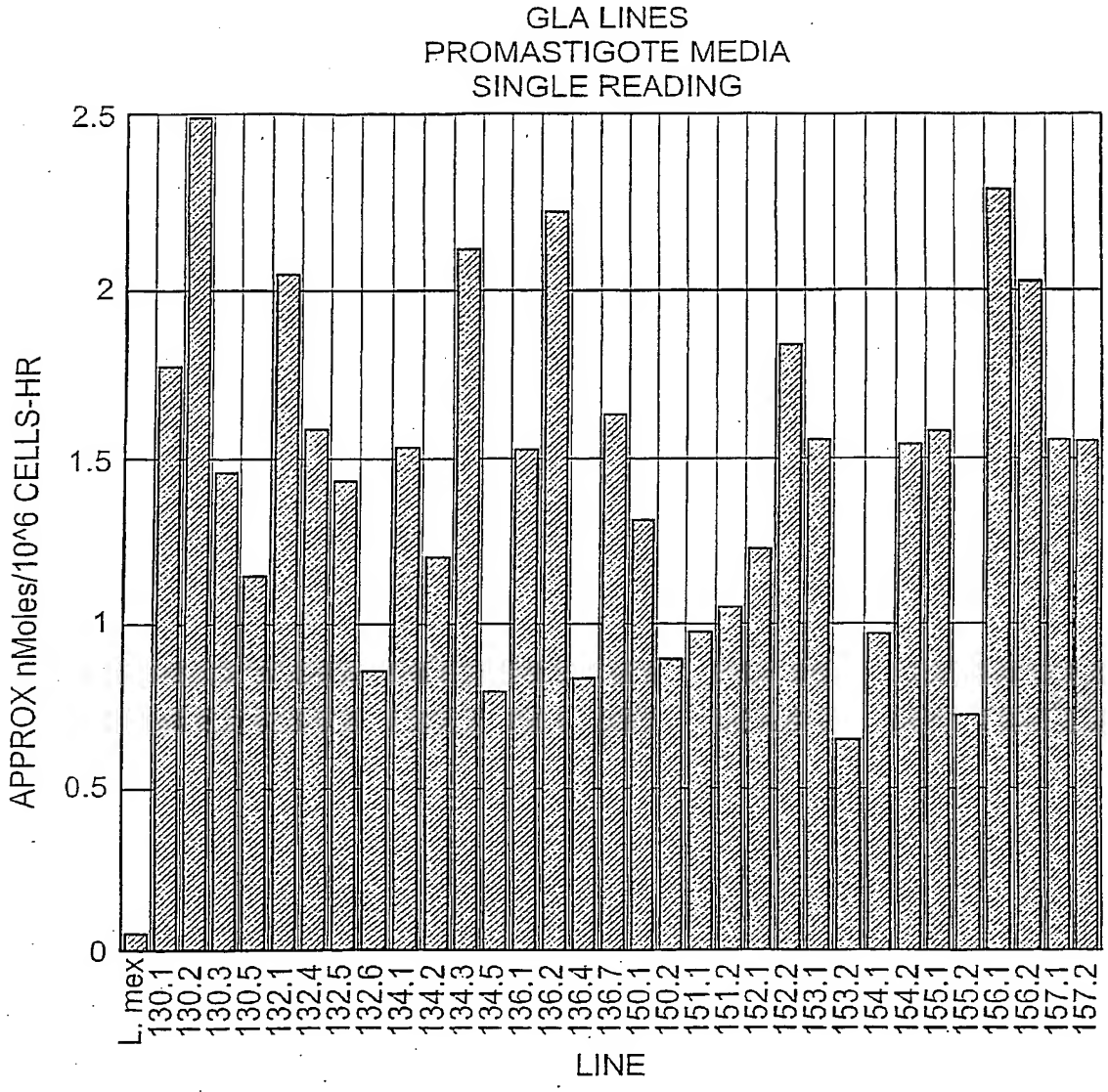


FIG. 8

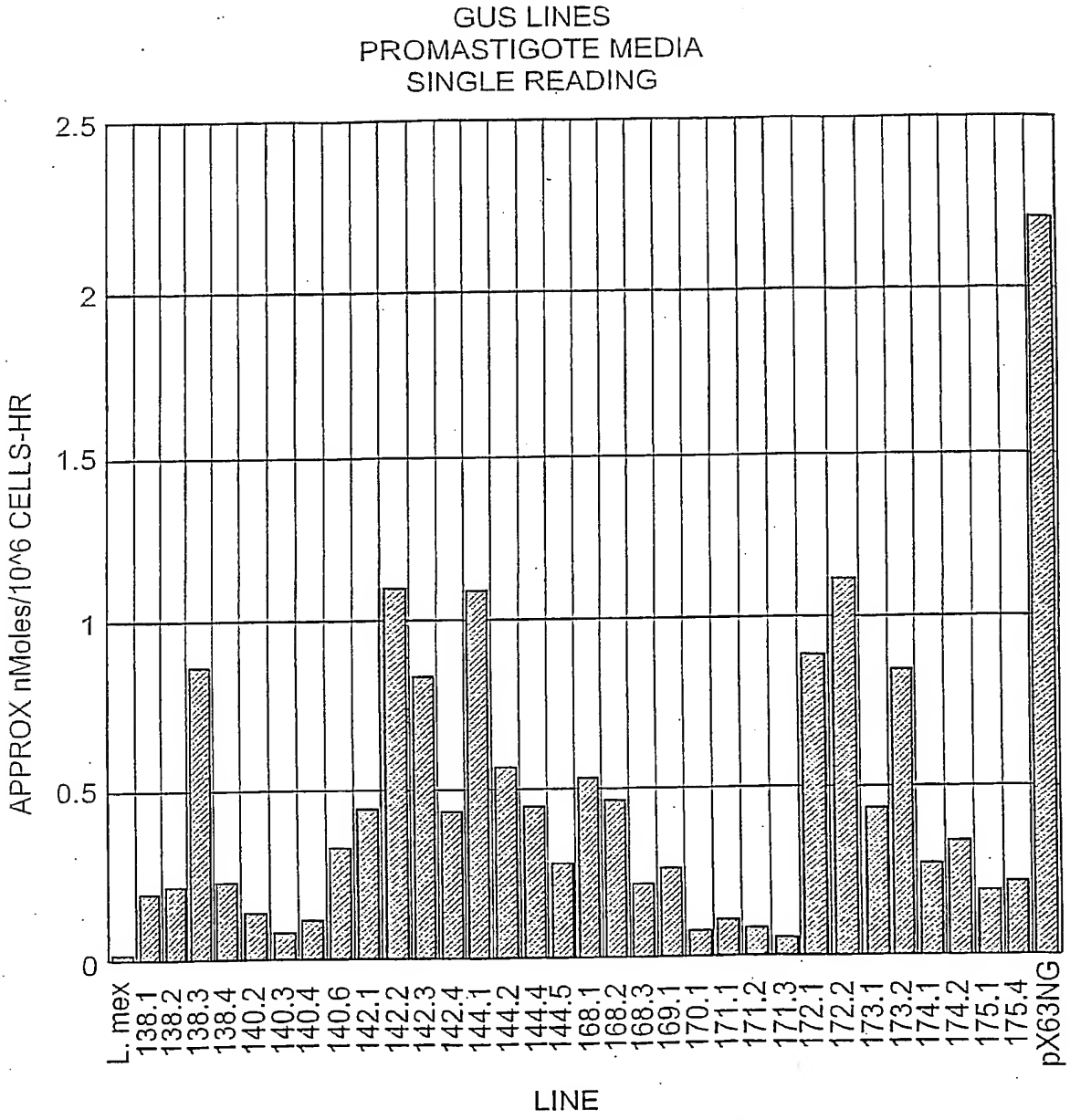


FIG. 9

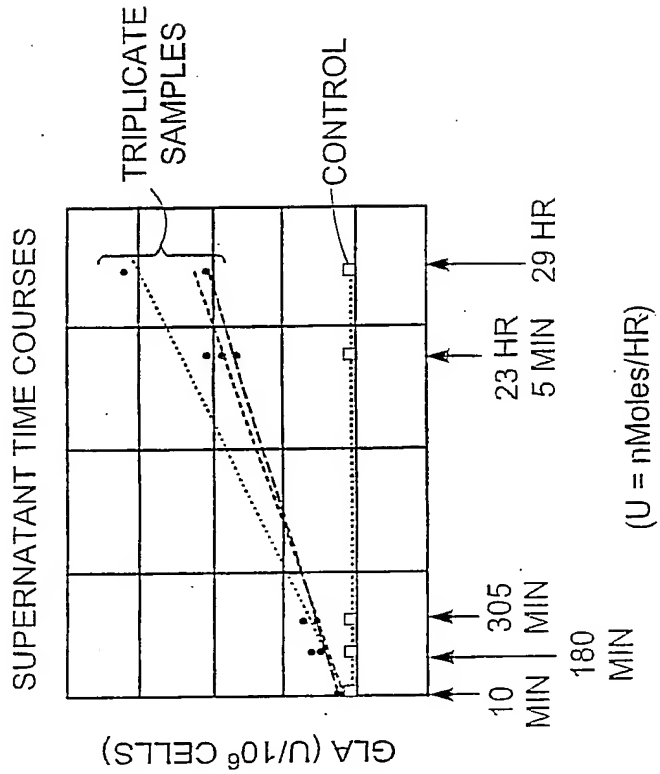


FIG. 10A-2

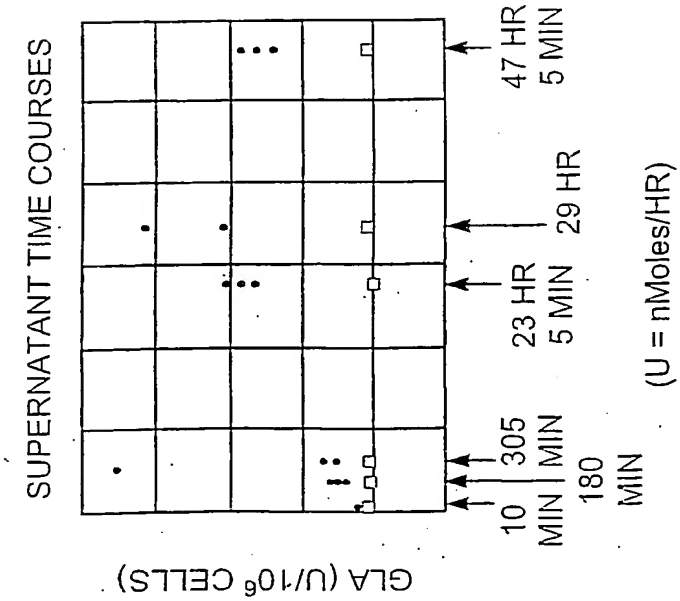


FIG. 10A-1

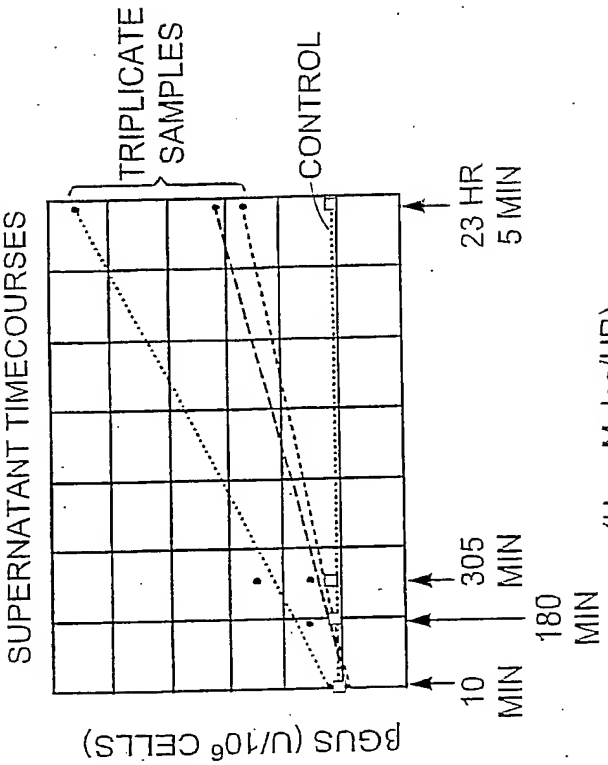


FIG. 10B-2

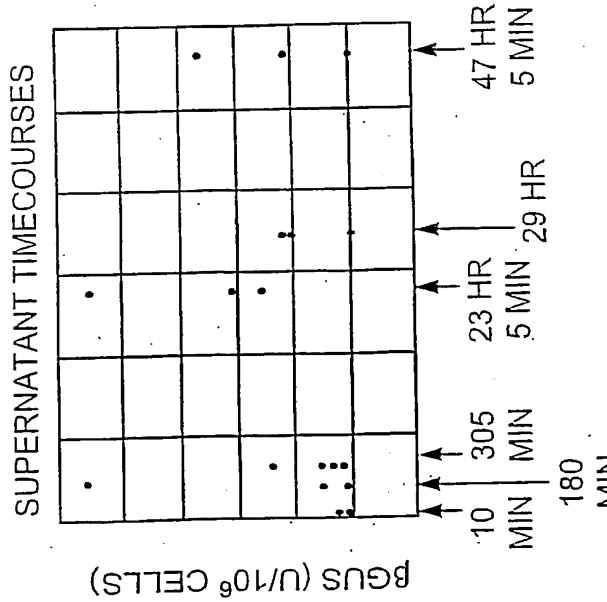


FIG. 10B-1

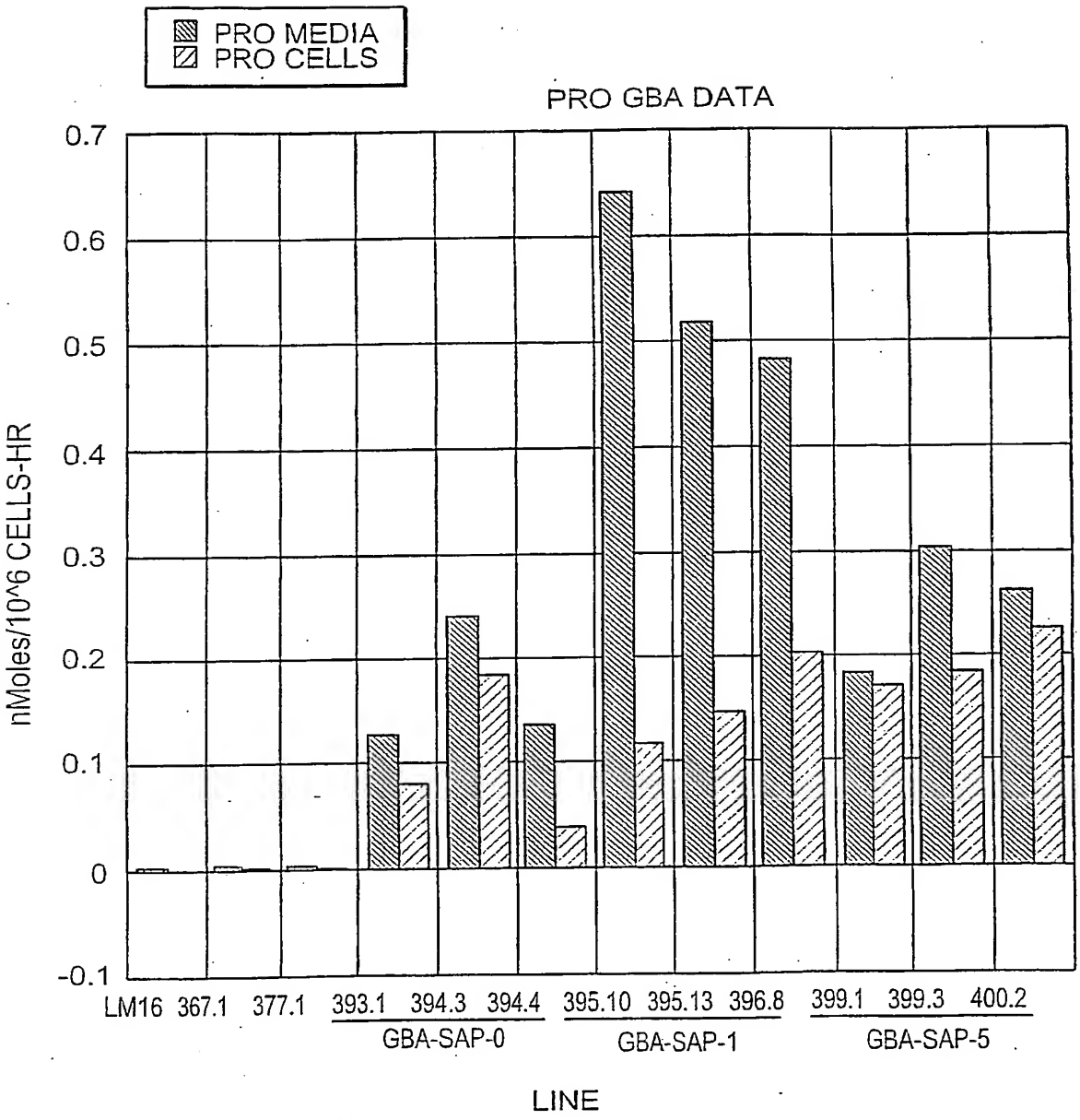


FIG. 11

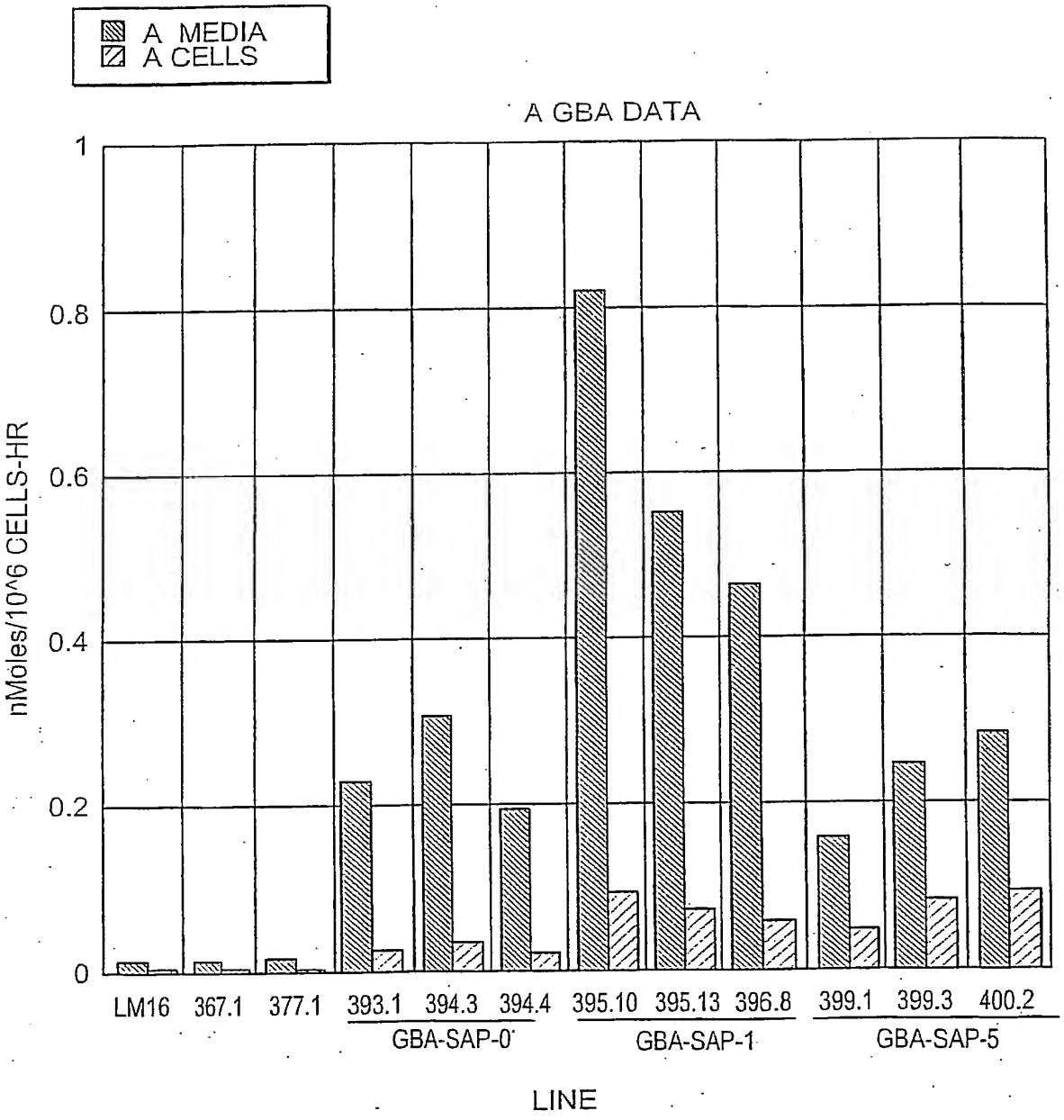


FIG. 12

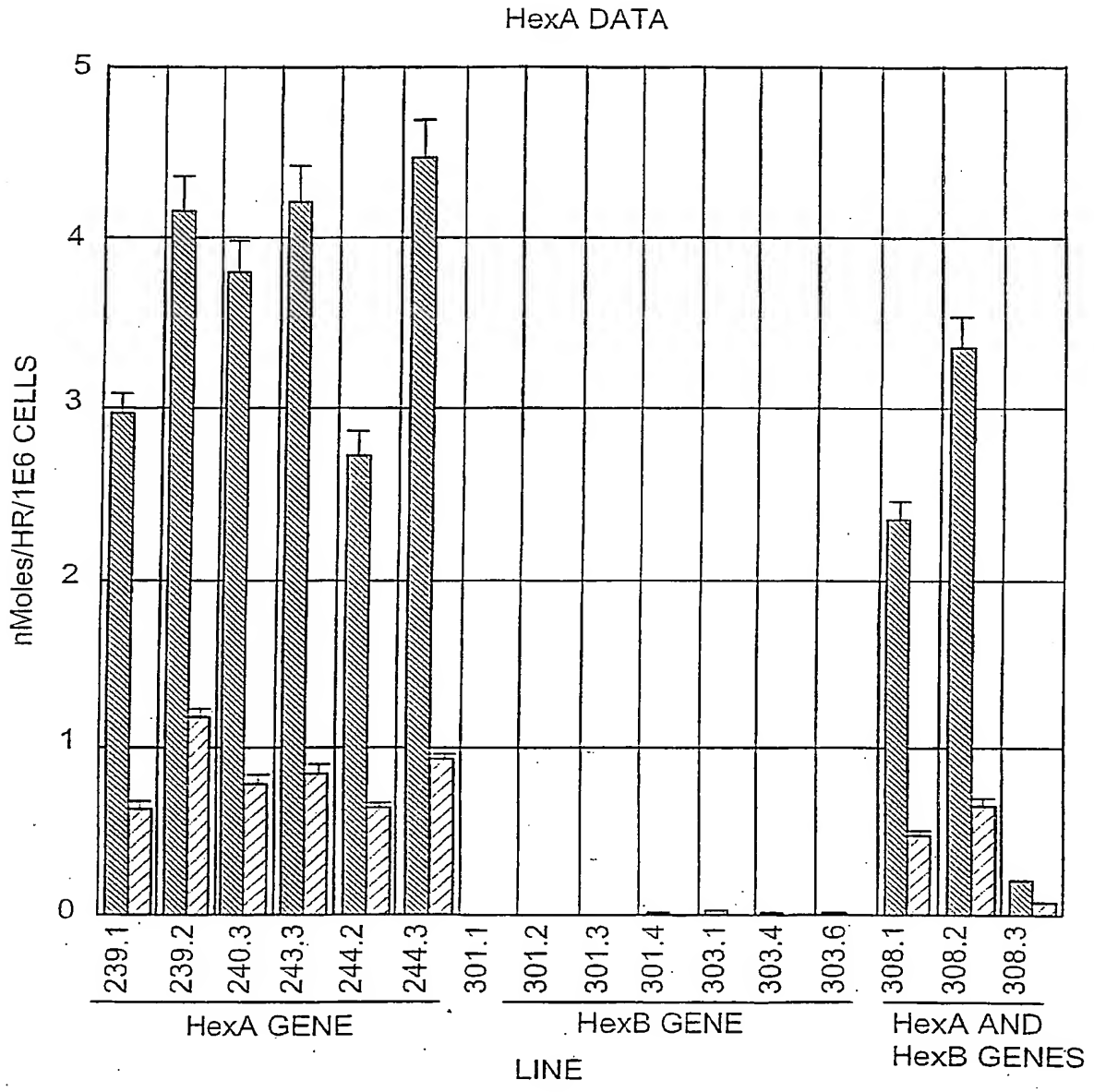


FIG. 13

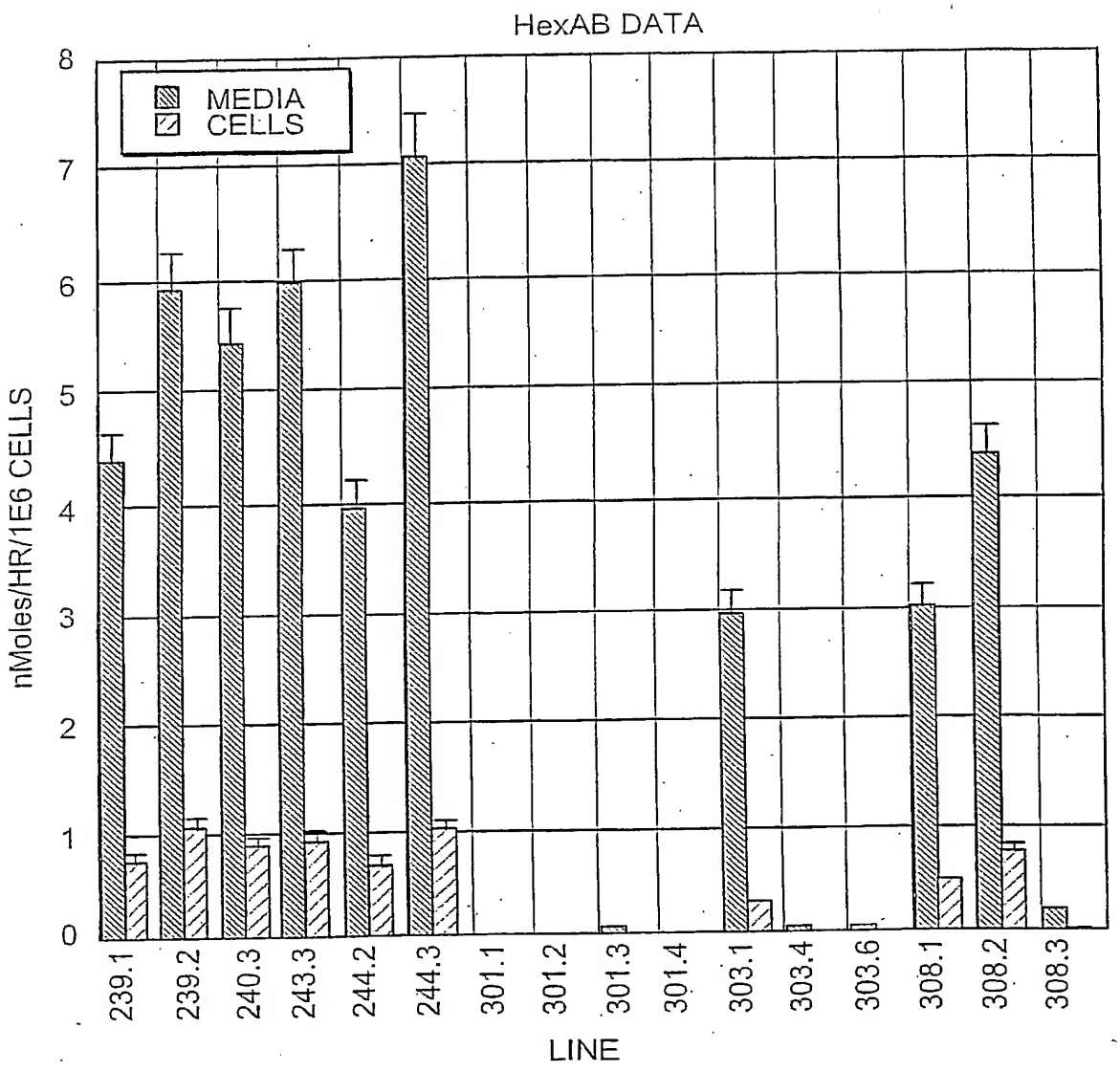


FIG. 14

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60/250,444	30 November 2000 (30.11.2000)	US
60/290,281	11 May 2001 (11.05.2001)	US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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— *with international search report*

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Previous Correction:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/044355 A3

(54) Title: PROTOZOAN EXPRESSION SYSTEMS FOR LYSOSOMAL STORAGE DISEASE GENES

(57) Abstract: Methods and devices are provided for expressing and secreting gene products from protozoa. The methods and devices are useful for expressing and isolating lysosomal storage disease enzymes from protozoa grown in culture, and particularly from trypanosomatids. The post-translational modification of isolated expression products can be adapted for administration to mammalian organisms. In addition, expression products can be isolated from serum free cultures thereby avoiding contamination by infectious agents such as prions. The methods and devices are also useful for delivering expression products such as lysosomal storage disease enzymes to mammalian organisms *in vivo*.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/44935

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/79 C12N15/56 C12N1/11 C12P21/02 A61K35/68
A61K38/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 11245 A (HARVARD COLLEGE ;SYMBIONTICS INC (US)) 19 March 1998 (1998-03-19)	1-3,7-12
Y	p. 6:1-7:25, 14:17-17:9, 49:6-6:20 example 9, claims	5,6

X	WO 00 58483 A (UNIV WASHINGTON ;BEVERLEY STEPHEN M (US)) 5 October 2000 (2000-10-05)	1-3,7-12
Y	abstract, p. 3:14,-11:28, 15:24-18:15, 21:19-22:33, claims	5,6

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 2003	09.05.03

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Puonti-Kaerlas, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VOTH BEVAN R ET AL: "Differentially expressed Leishmania major gp63 genes encode cell surface leishmanolysin with distinct signals for glycosylphosphatidylinositol attachment." MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 93, no. 1, 15 May 1998 (1998-05-15), pages 31-41, XP002227143 ISSN: 0166-6851 abstract, p. 39, fig.2	5,6
P,X	VACCARO D E: "Symbiosis therapy: the potential of using human protozoa for molecular therapy." MOLECULAR THERAPY: THE JOURNAL OF THE AMERICAN SOCIETY OF GENE THERAPY. UNITED STATES DEC 2000, vol. 2, no. 6, December 2000 (2000-12), pages 535-538, XP002227144 ISSN: 1525-0016 abstrac, p. 537-538	1-3,5-12
A	MEIKLE PETER J ET AL: "Prevalence of lysosomal storage disorders." JAMA (JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION), vol. 281, no. 3, 20 January 1999 (1999-01-20), pages 249-254, XP009003825 ISSN: 0098-7484	
T	BURKE B ET AL: "Macrophages in gene therapy: Cellular delivery vehicles and in vivo targets." JOURNAL OF LEUKOCYTE BIOLOGY, vol. 72, no. 3, September 2002 (2002-09), pages 417-428, XP009003789 September, 2002 ISSN: 0741-5400	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/44935

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 2 and 5-9 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 2, 3, 10-12 (all completely), 5-9 (partially)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 5-6 relate to a method wherein a regulatory element comprises a signal peptide.
The Sequence Listing as present in the description does not comply with WIPO standard ST 25 prescibed in the administrative instructions under Rule 5.2 PCT. The sequence listing has not been furnished on paper form or in machine readable form as provided for in the same instructions, nor have the applicants remedied the disclosed deficiencies within the time limit fixed in the invitation to PCT Rule 13ter.1.a. Consequently, the search has been carried out based on the trivial designations of said peptides used in the claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Inventions 1-3

Invention 1 (claim 1 complete, claims 7-9 partially)
A method for obtaining a lysosomal disease enzyme

Invention 2 (claim 2 complete, claims 5-9 partially)
A method for delivering a lysosomal disease enzyme to a patient

Invention 3 (claims 3,10,-12 complete, claims 5-6 partially)
A protozoan engineered to express a lysosomal disease enzyme

Invention 4 (claim 4 complete)

A method for obtaining a prion free expression protein preparation

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 01/44935

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9811245 A	19-03-1998	US	6020144 A	01-02-2000
		AU	742670 B2	10-01-2002
		AU	4479997 A	02-04-1998
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		JP	2001501462 T	06-02-2001
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		AU	3880300 A	16-10-2000
		EP	1165812 A2	02-01-2002
		JP	2002539836 T	26-11-2002
		WO	0058483 A2	05-10-2000